DOCTOR OF PHILOSOPHY

Investigating the role of CAR and PXR in the regulation of cytochrome P450s and other drug metabolising enzymes by anti-cancer drugs using novel humanised mouse models

Fraser, Emily Anne

Award date:
2013

DOCTOR OF PHILOSOPHY

Investigating the role of CAR and PXR in the regulation of cytochrome P450s and other drug metabolising enzymes by anti-cancer drugs using novel humanised mouse models

Emily Anne Fraser

2013

University of Dundee

Investigating the role of CAR and PXR in the regulation of cytochrome P450s and other drug metabolising enzymes by anti-cancer drugs using novel humanised mouse models

By

Emily Anne Fraser

A thesis presented to the University of Dundee for the degree of Doctor of Philosophy

December 2012

Contents

Chapter 4: Characterizing the interactions of aromatase inhibitors with PXR and CAR

Chapter 5: Final Conclusions

Acknowledgements

Firstly, I would like to thank my supervisors, Professor Roland Wolf, Dr Colin Henderson and Dr Robert Finn, for all their help and advice over the course of my PhD.

I would also like to thank everyone in the molecular pharmacology unit who has helped me throughout my studies. In particular, a huge thank you goes to Catherine Hughes, the stalwart of all my *in vivo* studies, and to Lesley McLaughlin, for all her help with the mass spectrometry and HPLC analysis, in addition to being my lab guru and keeping me sane. Thanks also to the other members of the mouse team (Jen Kennedy, Susanne van Schelven and Cheryl Wood) for all the help and support they've provided throughout my PhD. I would also like to thank Dr Douglas Vasey for his help with the histology and analysis, and Steph Sharp for helping me with some final experiments.

Grateful thanks to CXR Biosciences Ltd. and TaconicArtemis for allowing me to use their transgenic models in my study.

Finally I would like to thank my family for supporting my throughout my PhD. A huge thanks to Alex for cheering me up on the bad days and keeping me fed, and to Rory for not misbehaving too badly and letting me write this thesis. In particular, I'd like to thank my mum and mother-in-law for providing extended babysitting services, without which I could not have finished this project.

Candidates Declaration

I hereby declare that all results described in this thesis, unless otherwise stated, are entirely my own work. I further state that the composition of this thesis was performed by myself and none of the material has been submitted for any other degree. Lastly, I verify that all sources have been appropriately cited. This work was carried out in the Biomedical Research Institute, University of Dundee, under the supervision of Professor C. Roland Wolf and Dr Colin J. Henderson.

Emily Anne Fraser

Supervisor's Declaration

I certify that Emily Anne Fraser has completed nine terms of experimental research and has fulfilled the conditions of Ordinance 39, University of Dundee, such that he is eligible to submit the following thesis in application for the degree of Doctor of Philosophy.

Professor C.R. Wolf

Abstract

Nuclear receptor activation, particularly that of the pregnane X receptor (PXR) and constitutive androstane receptor (CAR), is increasingly recognised as a key determinant in the development of drug-drug interactions (DDIs) as a result of their key role in the transcriptional regulation of numerous drug metabolizing enzymes and drug transporters. PXR and CAR involvement in the regulation of the cytochrome P450 enzymes is of greatest concern, since these enzymes metabolise the majority of currently available therapeutics. Various methods are available to investigate the activation of these receptors in response to drug challenge, including reporter gene assays, primary human hepatocytes and transgenic mouse models. However, these models lack the sophistication to effectively assess receptor cross-talk, a key regulatory mechanism in the control of drug metabolism with the potential to impact the development of DDIs. Using a novel panel of PXR & CAR transgenic mouse models this study was designed to investigate the role of cross-talk between PXR and CAR in the metabolism and pharmacokinetics of commonly available pharmaceuticals, with particular emphasis on species-specific regulation.

This study has identified potential interactions with PXR and CAR following treatment of wild-type mice with cyclophosphamide, gefitinib, anastrozole and letrozole. Data from the PXR/CAR transgenic mouse panel has also provided evidence that the aromatase inhibitors, anastrozole and letrozole, interact with PXR and CAR in a species- and gender-specific manner. Cross-talk between these receptors plays a key role in the regulation of P450 expression and drug pharmacokinetics following treatment by these agents, although the elimination of these drugs appears to be primarily renal, in contrast to data derived from humans. Of particular note is the aromatase inhibitor-induced up-regulation of Cyp2b10 expression and activity observed in all models possessing a functional CAR moiety. A corresponding induction in CYP2B6 transcriptional activation has been confirmed in a novel reporter mouse model, indicating a potential DDI risk if co-administered with a drug requiring CYP2B6 for its metabolism, i.e. cyclophosphamide. These data therefore support the use of these models as a tool to dissect the regulatory cross-talk of these receptors in the control of drug metabolism, and thus to improve the assessment of DDI risk in the development of therapeutics.

Table of Figures

List of Tables

Abbreviations

ADR	Adverse drug reaction
AF-2	Activation function-2
AhR	Aryl hydrocarbon receptor
AI	Aromatase inhibitor
AMPK	AMP-activated protein kinase
AUC_{0-1440}	Area under the curve from time=0 to time=24 hrs (1440 mins)
$AUC_{0-\infty}$	Area under the curve from time=0 to time=∞
AUC_{0-t}	Area under the curve from time=0 to time=t
BFC	7-benzyloxy-4-trifluoromethylcoumarin
BMI	Body mass index
BQ	7-benzyloxyquinoline
BR	Benzoxyresorufin
C/EBPα	CCAAT/enhancer binding protein-α
CAR	Constitutive androstane receptor
CCRP	Cytoplasmic CAR retention protein
CDK-2	Cyclin-dependent kinase-2
CITCO	6-(4-chlorophenyl)imidazo-[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichloro-benzyl)oxime
CLEM4	Constitutive liver enhancer module of *CYP3A4*
C_{max}	Maximum concentration
C_{min}	Minimum concentration attained at steady state pharmacokinetics
CREBP	cAMP response element binding protein

DBD	DNA-binding domain
DBP	PAR-domain basic leucine zipper transcription factors albumin D-box binding protein
DDI	Drug-drug interaction
DR-	Direct repeat
E_2	Oestradiol
EFC	7-ethoxy-4-trifluoromethylcoumarin
EGR	Early growth response protein-1
ER	Ethoxyresorufin
ER	Oestrogen receptor
ER-	Everted repeat
ERK	Extracellular signal-related kinase
F	Bioavailability
FACE	Femara Anastrozole Clinical Trial
FDA	Food & Drug Administration
FSH	Follicle stimulating hormone
GH	Growth hormone
GMD	Geometric mean diameter
HFC	7-hydroxy-4-trifluoromethylcoumarin
HLF	Hepatic leukaemia factor
HNF	Hepatocytre nuclear factor
HQ	7-hydroxyquinoline
IL-11	Interleukin-11
I.V.	Intravenous

KO	Knock-out
LBD	Ligand binding domain
LH	Luteinizing hormone
MDMA	Methylenedioxymethamphetamine
MFC	7-methoxy-4-trifluoromethylcoumarin
MR	Methoxyresorufin
NCoR	Nuclear receptor co-repressor
NLS	Nuclear localization sequence
NR	Nuclear receptor
$OARE_{KI}$	Okadaic acid response element
P450	Cytochrome P450
PBREM	Phenobarbital responsive element module
PCN	Pregnenolone-16α-carbonitrile
PHH	Primary human hepatocytes
PK	Pharmacokinetic
PKA	Protein kinase-A
PKC	Protein kinase-C
P.O.	Administered orally
POR	Cytochrome P450 oxidoreductase
PP2A	Protein phosphatase-2a
PXR	Pregnane X receptor
PXRE	PXR response element
RIF	Rifampicin
RXRα	Retinoid X receptor-α

SMRT	Silencing mediator of retinoid and thyroid hormone receptor
SNP	Single nucleotide polymorphism
SRC	Steroid receptor co-activator
SV	Splice variant
T	Testosterone
$t_{½}$	Terminal half-life
TBST	Tris buffered saline-Tween
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TEF	Thyrotroph embryonic factor
t_{max}	Time at which C_{max} is achieved
TNF	Tumour necrosis factor
TRα1	Thyroid receptor-1α
UGT	UDP-glucuronosyltransferase
V_d	Volume of distribution
VDR	Vitamin D receptor
W/T	Wild-type
XREM	Xenobiotic response element
XRS	Xenobiotic response sequence

CHAPTER 1

INTRODUCTION

1.1 XENOBIOTIC METABOLISM AND ITS ROLE IN THE DEVELOPMENT OF DRUG-DRUG INTERACTIONS

1.1.1 DRUG-DRUG INTERACTIONS: INCIDENCE AND RISK FACTORS

One of the key issues associated with drug development is the accurate identification of drug-drug interactions (DDIs), which occur when one drug interferes with the function of another co-administered therapeutic. This interference can occur as a result of modulation of the pharmacokinetics, pharmacodynamics or pharmaceutical properties of an agent, resulting in altered drug disposition or activity (Riechelmann et al., 2005; Riechelmann and Krzyzanowska, 2006; Riechelmann and Saad, 2006; Riechelmann, 2007; Riechelmann and Krzyzanowska, 2007; Riechelmann et al., 2008; Riechelmann and Del Giglio, 2009). Importantly, this phenomenon has several serious clinical implications, being capable of modulating clinical efficacy and having the potential to cause increased toxicity as a result. Estimates of the frequency of DDIs in clinically relevant adverse drug reactions are limited, and significantly variable as a result of intra-population variation. A UK-based study investigating DDI incidence in a population of 18,820 subjects estimates that of 6.5% of the population admitted with adverse drug reactions (ADRs), 16.6% were as a direct result of drug-drug interactions (Pirmohamed et al., 2004), although other smaller studies indicate a large range of between 15-59% of ADRs being attributable to DDIs (Davies et al., 2009; Reis and Cassiani, 2011). Although this is a comparatively small proportion of the test population, when considering this result from a whole population perspective, it is clear that this issue will affect significant numbers of people, and therefore an understanding of the factors implicated in the development of DDIs is essential.

Numerous studies have demonstrated that the risk of DDIs is increased by four factors: Age, gender, number of co-morbidities and number of concurrently administered drugs (Pirmohamed et al., 2004; Riechelmann et al., 2005; Riechelmann and Krzyzanowska, 2006; Riechelmann and Saad, 2006;

Riechelmann, 2007; Riechelmann and Krzyzanowska, 2007; Riechelmann et al., 2008; Davies et al., 2009; Riechelmann and Del Giglio, 2009; Hu and Hayton, 2011; Miranda et al., 2011; Bucşa et al., 2012; Obreli-Neto et al., 2012) The concurrent administration of various drugs, also known as polypharmacy, is of particular concern, with the risk of DDIs increasing from 13% with 2 drugs, to 38% with 4 drugs, and exponentially increasing to 82% with 7 drugs (Goldberg et al., 1996; Buajordet et al., 2001). This is a particular problem in the elderly, with estimates suggesting that 13-92% of this population receive multiple concurrent therapies as a result of the increasing co-morbidity burden observed with age (Lees and Chan, 2011; Obreli-Neto et al., 2012). The elderly are therefore particularly vulnerable to the development of DDIs, with the effect being exacerbated by age-related decline in various physiological processes, including drug metabolism and renal elimination, which can themselves alter drug absorption and disposition (Obreli-Neto et al., 2012). It is also of prime importance in the treatment of complex diseases, in which the use of co-administration regimes is prevalent.

A therapeutic area of particular concern is that of oncology, in which not only are combination regimes of anti-neoplastic agents prevalent, but also regularly combined with treatments to ameliorate drug toxicity, in addition to those already being administered to the patient to treat co-morbidities. In addition to prescribed drugs, there is further potential for DDIs from over-the-counter remedies which are also commonly co-administered by the patient. Median estimates of the number of drugs taken in oncology patients range from 5-9 prescribed medications per patient (Hanigan et al., 2008; Puts et al., 2009; Cashman et al., 2010; Lees and Chan, 2011; Prithviraj et al., 2012), with the only study isolating over-the-counter drug use estimating frequency at 1.7 over-the-counter medicines per patient (Prithviraj et al., 2012). According to the data presented by Goldberg *et al.* (1996), this puts this population at high risk of DDIs, and this escalates further when considering that cancer is more prevalent in the elderly population. Various studies have indicated that approximately one third of ambulatory cancer patients are at risk of potential DDIs (Riechelmann, 2007; Riechelmann and Krzyzanowska, 2007; Riechelmann et al.,

2008; Riechelmann and Del Giglio, 2009), although this figure appears to be higher in those populations with brain tumours/metastases as a result of prescribed anti-convulsant drugs (Riechelmann and Del Giglio, 2009). Of these interactions, 87% are related to medications prescribed alongside anti-neoplastic therapies, both to support chemotherapy and to treat co-morbidity. The most commonly interacting drugs include aspirin, warfarin, anti-depressants and anti-convulsants (Riechelmann, 2007; Riechelmann et al., 2008; Riechelmann and Del Giglio, 2009). However, this information is theoretical, being based on computer simulation to identify potential interactions between prescribed drug pairs. The clinical prevalence of these interactions is still poorly understood, although a Norwegian study has demonstrated that 4% of all deaths in a cancer population were as a direct result of fatal adverse drug interactions (Buajordet et al., 2001). Clearly, the clinical evidence base must be increased before the full extent of the problem in oncology treatment can be understood. However, given the narrow therapeutic indices of the majority of anti-neoplastic agents, an interaction could have potentially fatal consequences through a reduction in clinical efficacy or increase in toxicity.

To this point, only traditional pharmaceuticals have been discussed with respect to the risk of DDIs. However, a potentially more serious issue relates to the interaction of therapeutics with unregulated sources of interacting chemicals, primarily complementary therapies, such as herbal medicines, but also various chemicals found in food, including vitamins and flavonoids. Numerous pre-clinical studies have demonstrated the ability of a number of herbal and dietary constituents to cause drug interactions, frequently through modulation of drug pharmacokinetics (Williamson, 2003; Rockwell et al., 2005; Mandlekar et al., 2006; Meijerman et al., 2006; Tirona and Bailey, 2006; Izzo and Ernst, 2009; He et al., 2010; Kennedy and Seely, 2010; Mason, 2010; Tarirai et al., 2010; Izzo, 2012; Won et al., 2012). However, published clinical evidence regarding their use and safety in oncology treatment remains comparatively scarce for the majority of herbal medicines, with the best evidence base being available for St. John's Wort (Mannel, 2004; Zhou and Lai, 2008; Borrelli and

Izzo, 2009; Izzo and Ernst, 2009; Kennedy and Seely, 2010; Tarirai et al., 2010; Olaku and White, 2011; Izzo, 2012). The lack of clinical evidence base makes it challenging for physicians to correctly advise on the use of these chemicals, as well as to identify potential herb-drug interactions when they present. This burden is increased by the finding that 30-60% of herbal supplement users will not tell a health professional that they are taking herbal medicines (Werneke et al., 2004; Engdal et al., 2008; McLay et al., 2011). Investigations into the cause of this under-reporting suggest that it is primarily due to a lack of understanding of the risks of herbal supplements, with a small scale random sample indicating that the common perception is that they are safer than conventional medicine because they are natural, and thus less likely to cause side effects, interact with other drugs or result in dependency (Lynch and Berry, 2007; Dunne, 2009). One study identified fewer than 50% of herbal supplement users that understood that herbal medicines could interact with conventional drugs (McLay et al., 2011). This lack of education and under-reporting is of particular concern in the cancer population, in which not only is there a high risk of polypharmacy, but also a higher probability of herbal medicine use. Estimates of the frequency of herb and dietary supplement use in cancer populations are extremely variable, being affected by numerous factors including the country the study is performed in, type of cancer and gender. In a general UK cancer population, estimates of herbal medication use range between 3.1-21.8% in adults (Gratus et al., 2009a; Gratus et al., 2009b; Damery et al., 2011). However, a study in a general cancer population in Norway assesses the frequency as 46%, possibly reflecting a greater acceptance of herbal therapy in Norwegian society (Engdal et al., 2008). The incidence of herbal preparation use is highest in the female breast cancer population with frequency being reported as 22.7-38.4% in UK populations (Damery et al., 2011; McLay et al., 2011). In the majority of cases, herbal supplements were commenced without obtaining any professional advice, either from a physician or herbalist (Damery et al., 2011; McLay et al., 2011), and therefore it is evident that unexpected DDIs caused by un-reported herbal medicine therapy could be a significant problem in cancer populations. The potential for DDIs is therefore

significant in this subset of the population, but urgently requires further study in large scale clinical trials before it can be effectively managed in the clinic.

From these studies, it is evident that oncology patients are at a high risk of DDIs, primarily as a result of polypharmacy. However, systematic analysis of DDI prevalence in clinical populations is limited. More complete clinical analysis of this phenomenon is therefore essential to provide oncologists with the best information on which to base clinical decisions regarding treatment regimes and to aid in the recognition of adverse drug events. In order to initiate well-directed clinical trials, pre-clinical *in vitro* and *in vivo* data is required to identify the molecular determinants of drug interactions, and thus the relative likelihood of a clinically relevant DDI occurring. The molecular basis of DDIs will now be considered.

1.1.2 Drug-drug Interactions: Role of the Cytochrome P450 system

DDIs can arise as a result of various factors, both related to the patient receiving the treatment, e.g. age and gender, and to the drug being administered (Hu and Hayton, 2011). Variations in any process linked to the absorption and disposition of a drug can result in DDIs, including physicochemical changes, such as variations in gastric pH and emptying time, and changes in drug pharmacodynamics. However, of particular concern are those interactions that occur as a result of pharmacokinetic interference, in which the metabolism of one drug is changed as a result of interactions between a co-administered agent and various proteins associated with drug metabolism. DDIs can occur at any level of drug metabolism (Phase I, Phase II or drug transport). However, of particular concern are interactions with the cytochrome P450 (P450) enzyme system which is responsible for the metabolism of the majority of pharmaceuticals (Lee et al., 2006; Zhang et al., 2010).

Cytochrome P450s are the key constituents of Phase I metabolism, being involved with the metabolism of a range of endobiotic and xenobiotic substances (Plant, 2007; Omiecinski et al., 2011b; Singh et al., 2011). This

enzyme group is able to catalyse numerous reactions, including *N*- and *O*-dealkylation, aliphatic & aromatic hydroxylation, *N*- and *S*-oxidation, deamination, *C*-hydroxylation and epoxide formation (Lamb et al., 2007; Omiecinski et al., 2011b), with the primary aim being to generate a reactive side-group for downstream Phase II metabolism (Gibson and Skett, 2001). All reactions are catalysed by the haem core (ferriprotoporphyrin-9), located within the hydrophobic substrate binding pocket of the enzyme, in the presence of cytochrome P450 oxidoreductase (POR) (Henderson et al., 2003; Lamb et al., 2007; Coleman, 2010). Although 102 functional- and 88 pseudo-genes have been identified in the mouse genome, the total number of P450 genes identified in humans is 57 (Nelson et al., 2004; Plant, 2007; Singh et al., 2011). Of these genes, the most important from the perspective of xenobiotic metabolism are the members of the CYP1, CYP2 and CYP3 families, with the 7 major isoforms (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) having been associated with the metabolism of more than 90% of currently available therapeutics (Zhang et al., 2010; Pinto and Dolan, 2011; Singh et al., 2011). It is the prevalence of P450-mediated drug metabolism which results in the predisposition to DDIs caused by P450-interaction (Lee et al., 2006). The importance of the P450 enzyme system is further highlighted by recent FDA draft guidance for industry regarding the design of DDI trials, which recommends that the assessment of a drug's ability to induce P450s is the first priority in assessing the risk of pharmacokinetic interactions (Zhang et al., 2010; FDA, 2012). Importantly, they recommend that initial analysis should encompass the induction of CYP1A2, CYP2B6 and CYP3A *in vitro* prior to assessment of other P450s, including CYP2C8, CYP2C9, CYP2C19 and CYP2D6, progressing to *in vivo* analysis if positive results are identified. Of particular concern to DDI studies is modulation of CYP3A4 activity and expression, and therefore this enzyme will be used to demonstrate risks caused by polypharmacy and the main causes of P450-mediated DDIs.

CYP3A4 is the dominant member of the *CYP3A* gene family in the majority of humans, accounting for 30-50% of total liver P450 content, and being associated with the metabolism of up to 60% of current therapeutics (Plant,

2007). It is also a key enzyme in first-pass drug metabolism, being expressed in the small intestine (van Herwaarden et al., 2009; Van Waterschoot et al., 2009). Any interference with this enzyme therefore has significant potential to cause DDIs through the modulation of the pharmacokinetic parameters of a co-administered drug which also relies on metabolism by CYP3A4. Substrates of CYP3A4 are wide ranging, and include commonly prescribed medications, such as statins (simvastatin, lovastatin, atorvastatin), over-the-counter medications, such as codeine, herbal medicines, such as St Johns' Wort (specifically its constituent, hyperforin), and dietary constituents, such as theophyllines and caffeine (Manzi and Shannon, 2005; Hisaka et al., 2010; Hokkanen et al., 2011). Importantly, many commonly used anti-cancer agents are substrates of CYP3A4, including several tyrosine kinase inhibitors (imatinib, gefitinib, erlotinib, nilotinib, dasatanib), the vinca alkaloids (vincristine, vinblastine and vinorelbine), the topoisomerase I inhibitor, irinotecan, the mitotic inhibitor, paclitaxel, the aromatase inhibitors, anastrozole, letrozole and exemestane, and the oestrogen receptor antagonist, tamoxifen (Sioufi et al., 1997a; Desta et al., 2004; Manzi and Shannon, 2005; Scripture et al., 2005; Sanford and Plosker, 2008; Murai et al., 2009; Hisaka et al., 2010; Ingle et al., 2010; Kamdem et al., 2010; Lazarus and Sun, 2010; Desta et al., 2011). The potential for DDIs mediated by CYP3A4 during cancer therapy is therefore significant, especially when considering the prevalence of polypharmacy regimes, although the effects are dependent on the type of interaction and the mechanism of the drug in question. For instance, a DDI which induces enzyme activity could either increase the toxicity or clinical effectiveness of a pro-drug which requires the P450 in question for activation, or substantially reduce the clinical effectiveness of an active drug through excessive metabolism. Clearly the effects would be reversed if the DDI inhibits enzyme activity. It is therefore vital that the mechanism of a potential DDI is known, together with the metabolic nature of the drug, so that potential clinical effects can be effectively assessed.

Interactions between drugs can be considered as two distinct types - 1) those that rely on enzyme inhibition, and 2) those that are caused by modulation of enzyme expression. DDIs caused by enzyme inhibition are the best

characterised, and are the result of direct interaction between the enzyme and an inhibitory substance, resulting in reduced enzyme activity, and thus reduced metabolism. These DDIs are observed rapidly following drug administration as a result of this direct interaction, with their longevity being dependant on type of inhibition (competitive, non-competitive or mechanism-based). Substances that are known to strongly inhibit CYP3A4 activity include azoles (ketoconazole, itraconazole, voriconazole), macrolide antibiotics (clarithromycin, telithromycin, erythromycin), anti-retroviral protease inhibitors (ritonavir, saquinavir, nelfinavir, indinavir, atazanavir) and grapefruit juice (constituent responsible not yet determined) (Manzi and Shannon, 2005; Hisaka et al., 2010). Extreme caution is therefore required before initiating combination treatment regimes involving any of these chemicals in combination with other drugs that are metabolised by CYP3A4. Although this type of DDI is clearly of importance, it shall not be discussed further because it is outwith the remit of this study. However, further information regarding inhibition-based interactions with P450s can be found in the following reviews (Fontana et al., 2005; Bachmann, 2006; Ghanbari et al., 2006; Hisaka et al., 2010; Lutz and Isoherranen, 2012).

The second mechanism underlying DDIs, and the one most relevant to this study, are those DDIs mediated by interference between xenobiotics and gene transcription. Of particular concern are those that are mediated by interaction with the transcription factors and nuclear receptors regulating metabolic proteins, such as the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR). Activation or repression of the activity of these regulators can have significant effects on downstream pharmacokinetics as a result of drug-induced modulation of target gene expression. The indirect modulation of gene transcription means that this type of interaction is unlikely to be observed until several days following drug administration, once protein induction/repression has stabilized. The importance of these regulators in the development of DDIs is highlighted by the recommendations made in the FDA draft guidance regarding DDIs (Zhang et al., 2010; FDA, 2012). The initial assessment of CYP1A2, CYP2B6 and CYP3A

induction recommended in the guidance provides two important indications. Firstly, it provides an assessment of the potential for DDIs mediated by these three isoforms, all of which have been implicated in the metabolism of numerous drugs. However, perhaps more importantly, they also give an indication of key interactions at a regulatory level, being prototypically induced by activation of AhR (CYP1A2), CAR (CYP2B6) and PXR (CYP3A4). The modulation of PXR is of particular concern, being a key regulator of CYP3A4 (Moore and Kliewer, 2000; di Masi et al., 2009). However, the role of CAR as a mediator of DDIs is also evolving, with the increasing identification of interacting chemicals (Küblbeck et al., 2011a; Küblbeck et al., 2011b; Omiecinski et al., 2011a; Lynch et al., 2012). Both receptors are also known to operate in a co-operative manner to regulate target genes, providing robust control of numerous metabolic proteins, and making these nuclear receptors two of the key regulators of xenobiotic metabolism. In order to understand the role of these nuclear receptors in the development of DDIs, the following section will therefore consider PXR and CAR, and their role in the regulation of xenobiotic metabolism, in detail.

1.2 PXR AND CAR ARE KEY REGULATORS OF DRUG METABOLISM

1.2.1 PXR

The pregnane X receptor (PXR, also known as SXR, PAR and NR1I2), named for its activation by pregnenolone derivatives, is a highly promiscuous orphan nuclear receptor known to be a key regulator of xenobiotic metabolism. Its identification was comparatively recent, with both mouse and human orthologues being discovered in 1998, although orthologues have now been identified in other mammalian species, in addition to chicken and zebrafish (Blumberg et al., 1998; Kliewer et al., 1998; Krasowski et al., 2011). It is primarily expressed in liver and small intestine, although lower levels have also been detected in other tissues, including heart, colon, stomach and some brain regions (Lamba et al., 2004). This reflects its primary role as a xenosensor,

controlling the expression of various genes involved with drug metabolism, and in particular CYP3A4 **(Table 1.2.1)**.

Phase	Gene	PXR	CAR	Reference
Phase I	*CYP1A2*	↑	↑*	*(Yoshinari et al., 2010)
	CYP1B1	↑	↔	(Tirona et al., 2004)
	CYP2A6	↑*	↑	*(Itoh et al., 2006)
	CYP2B6	**↑**	**↑**	
	CYP2C8	↑	↑	(Ferguson et al., 2005)
	CYP2C9	↑	↔	(Sahi et al., 2009)
	CYP2C19	↑	↑	
	CYP3A4	**↑**	**↑**	
	CYP7A1	↓*	↓†	*(Rezen et al., 2010)†(Miao et al., 2006)
	CYP8B1	↓*	↓P†	*(Rezen et al., 2010); †(Beilke et al., 2009)
	CYP24A1	↑	↑	(Moreau et al., 2007)
Phase II	*UGT1A1*	↑	↑	(Bock, 2010)
	UGT1A3	↑		(MacKenzie et al., 2010; Bock, 2011)
	UGT1A4	↑		(MacKenzie et al., 2010; Bock, 2011)
	UGT1A6	↑		(MacKenzie et al., 2010; Bock, 2011)
	SULT2A1	↑		(Saini et al., 2004; Echchgadda et al., 2007; Bock, 2010)
Phase III	*MDR1*	↑	↑	(Klaassen and Aleksunes, 2010)
	MRP2	↑		
	MRP3	↑		

Table 1.2.1: Example human target genes of PXR and CAR involved with xenobiotic metabolism

*Adapted from Fraser et al. (2012). ↑=induced, ↓=repressed, ↔=no change/basal expression, P=putative interaction in humans. Bold indicates strong induction. Unless otherwise stated, data was extracted from di Masi et al. (2009). */† indicates relevant reference in final column.*

Class	Compound	PXR	CAR	References
Prototypical	Rifampicin	++		(Kobayashi et al., 2005;
	CITCO		++	Duret et al., 2006; Sinz
Drug	Atorvastatin	+	+	et al., 2006; Stanley et
	Simvastatin	++	+	al., 2006; Staudinger et
	Camptothecin	+		al., 2006; Kőhalmy et
	Omeprazole	+	+	al., 2007; Ricketts et al.,
	Paclitaxel	+		2007; Satsu et al.,
	Methadone	+	+	2008; di Masi et al.,
	Clotrimazole	-	-	2009; Hernandez et al.,
	Ketoconazole	-	-	2009a; Tolson et al.,
	Mifepristone	+		2009; Chen et al., 2010;
	Warfarin	+		Dong et al., 2010;
Herb	Hyperforin	++		Rulcova et al., 2010;
	Cryptotanshinone	+		Yao et al., 2010; Hoffart
	Artemisinin	+	+	et al., 2011; Howe et
	Ginkgo biloba	+	+	al., 2011; Küblbeck et
	Schisandrin A-C	+		al., 2011a; Küblbeck et
Dietary	Cafestol	+		al., 2011b; Marino et
	β-Carotene	+		al., 2011; Fraser et al.,
	Flavonoids	+	+	2012)
Endogenous	Androstenol		-	
	Dehydroepiandrosterone	+	+	
	17β-Estradiol	+	+	
	Vitamin K2	+		
Environmental/	Phthalic acid (DHEP)	+	+	
Industrial	Nonylphenol	+	+	

Table 1.2.2: Example modulators of human PXR and CAR

Blank spaces indicate no data is available. ++, very strong agonist; +, agonist; –, antagonist (or inverse agonist, CAR only).

However, it also has many physiological functions, including in inflammation, angiogenesis and control of energy, cholesterol and bile acid metabolism (Stanley et al., 2006; Staudinger et al., 2006; di Masi et al., 2009). It therefore has an extremely wide substrate specificity, encompassing various endogenous and exogenous chemicals **(Table 1.2.2)**. It is this promiscuity, combined with the potential breadth of downstream target gene induction, which makes it such a significant concern in the design of novel therapeutics (Moore and Kliewer, 2000; Stanley et al., 2006; Fraser et al., 2012). Although not specifically recommended in the FDA draft guidance, the use of PXR reporter assays to assess PXR activation potential of a novel drug is commonly used by the pharmaceutical industry as part of early pre-clinical development to eliminate chemicals with an unacceptable risk of DDIs at an early stage (Lin, 2006; Sinz et al., 2006; FDA, 2012). Common methodologies used to assess nuclear receptor activation and its effects will be discussed in *Chapters 3 & 4*.

The wide substrate specificity of PXR is a result of the large, highly flexible ligand binding domain (LBD) **(Figure 1.2.1)**. It consists of a three-layered α-helical sandwich and a five-stranded anti-parallel β sheet, unique to PXR (Watkins et al., 2001; Timsit and Negishi, 2007; di Masi et al., 2009). The activation function-2 helix (AF-2; helix 12), essential for transcriptional activation, is packed against the body of the receptor at the rear of the LBD (Watkins et al., 2001; di Masi et al., 2009). Other critical features required for receptor promiscuity are a 4 amino acid turn, known as the α2 helix, which appears to be involved with ligand entry and exit from the binding pocket by opening an access pathway, and the replacement of the α6 helix with a conserved flexible loop (residues 309-321). Together these features allow the binding pocket to expand from its resting 1150 $Å^3$ to more than 1600 $Å^3$ when ligand bound (Watkins et al., 2001; Watkins et al., 2003; Timsit and Negishi, 2007). This allows PXR to be activated by large molecules, such as the macrolide antibiotic rifampicin (RIF), using an induced fit mechanism, with ligands being able to "test" multiple binding orientations within the LBD to obtain maximal activation (Watkins et al., 2003; Chrencik et al., 2005; Orans et al., 2005). Of the 28 amino acids that line the predominantly hydrophobic LBD,

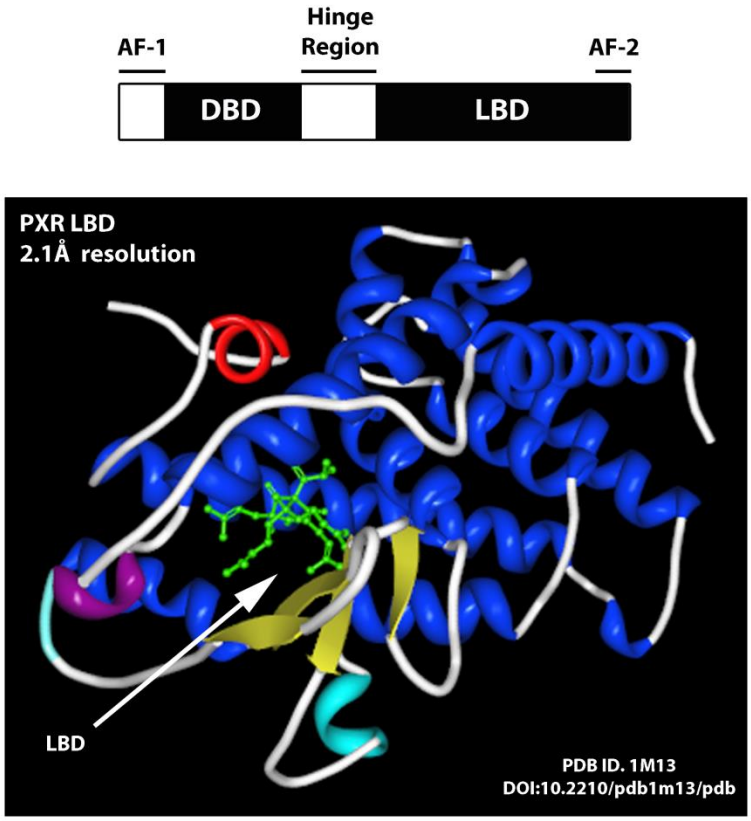


Figure 1.2.1: Structural representations of hPXR

Ribbon depicts domain structure of hPXR, highlighting activation function-1 (AF-1), DNA binding domain (DBD), hinge region, ligand binding domain (LBD) and activation function-2 (AF-2). Crystal structure of the PXR LBD in complex with hyperforin (2.1Å resolution) adapted from PDB ID: 1M13 (Watkins et al.*, 2003). Light blue helix = α2 helix, purple helix = conserved flexible loop replacing α6 helix, red helix = AF-2 (helix 12). 3D protein structure adapted using RCSB PDB Protein Workshop 4.1.0 (Moreland et al., 2005).*

only Met243, Ser247, Gln285, Trp299, His407 and Phe420 commonly interact with ligands (Watkins et al., 2001; Ekins et al., 2007; Timsit and Negishi, 2007). The importance of these amino acids has been demonstrated by site-directed mutagenesis, with even minor changes in the LBD resulting in significant effects on ligand-mediated PXR activation (Watkins et al., 2001; Watkins et al., 2003). This is of particular importance when considering variations in PXR activation and downstream gene protein (CCRP) and HSP90 (Kawana et al., 2003; Squires et al., 2004). Upon activation, PXR dissociates from this complex by an unknown mechanism and translocates to the nucleus as a result of the

recognition of nuclear localization signals by importin-α proteins located at the nuclear membrane **(Figure 1.2.2)** (Kawana et al., 2003).

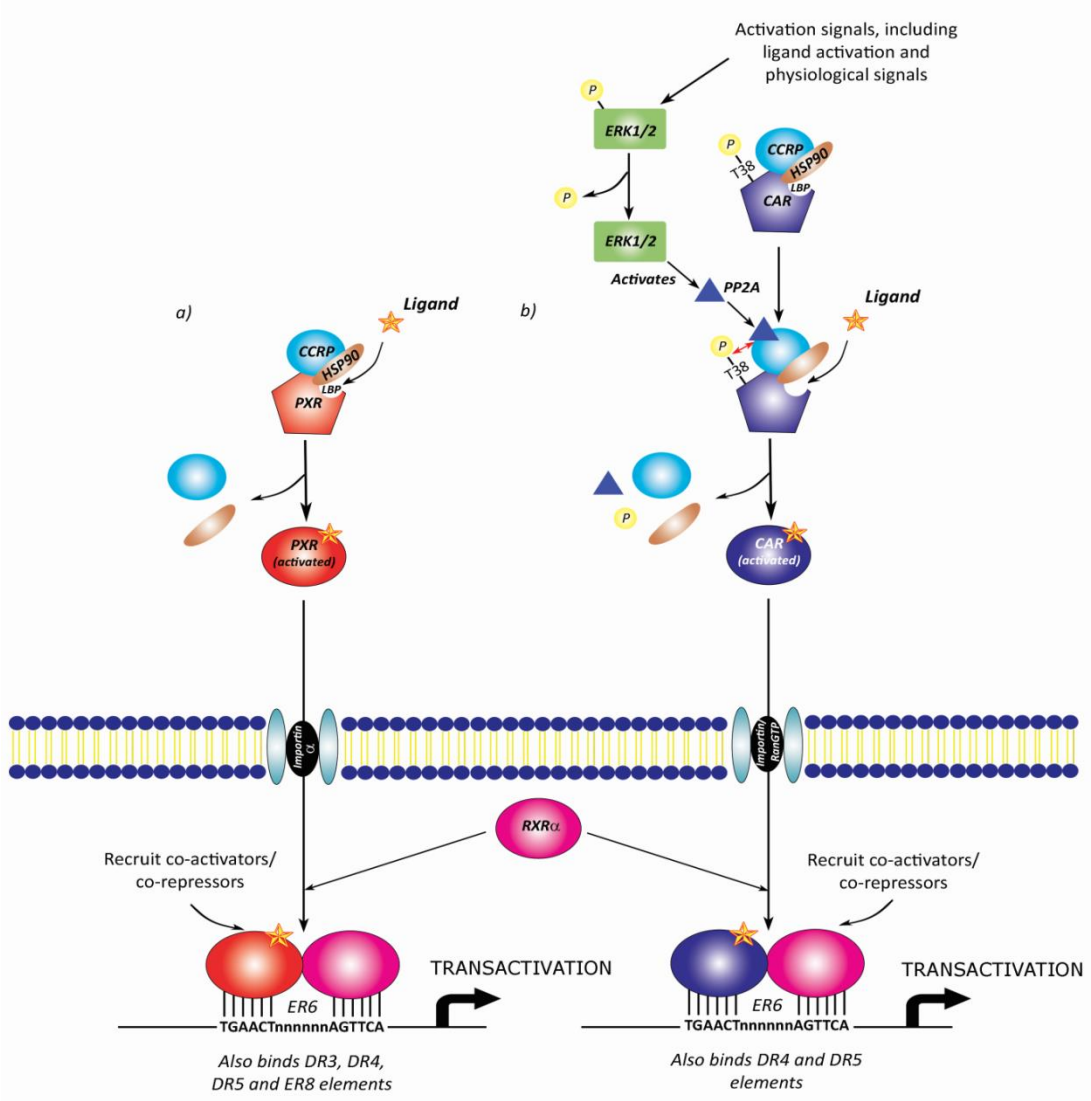


Figure 1.2.2: Activation protocol for PXR (a) and CAR (b)

Adapted from Fraser et al. (2012)

PXR possesses two nuclear localization signals (NLS), with the activity of each being dependent on the mechanism of PXR activation. The first is a bipartite nuclear localisation signal with the sequence RRXXKR---RXXRR, and is vital for ligand-independent nuclear translocation (Kawana et al., 2003). The second is known as the xenochemical response signal (XRS), with the sequence MXXLXXL, and is located in the C-terminus of the LBD (Kawana et al., 2003; Squires et al.,

2004). This signal has been associated with ligand-dependent nuclear translocation. Following nuclear translocation, PXR heterodimerizes with its binding partner, the retinoid X receptor-α (RXRα), prior to binding response elements in its target genes. The favoured binding sites for the PXR/RXRα heterodimer in target genes are two AG(G/T)TCA half sites separated by 3 nucleotides in a direct repeat formation (DR-3), and two everted repeats of this half site separated by 6 nucleotides (ER-6) (Stanley et al., 2006; di Masi et al., 2009). However, they are able to bind other recognition sites, such as DR-4, DR-5 and ER-8 motifs, although with lower affinity (Kliewer et al., 2002; Fraser et al., 2012). This capability is of key importance in transcriptional control, because it forms the basis of crosstalk between nuclear receptors, such as PXR and CAR.

The recruitment of co-activators/co-repressors to the target gene is essential to nuclear receptor-mediated gene transcription. The function of these proteins is to achieve structural changes in the conformation of the nuclear receptor which either promote or repress transcription. Examples of co-activators/co-repressors for PXR and CAR are given in **Table 1.2.3**. Among the co-activators recruited by PXR are the p160/steroid receptor co-activator (SRC) family. The function of these proteins is to recruit histone acetyltransferase complexes, such as cAMP response element binding-protein (CREBP)/p300, which modify DNA structure to permit RNA polymerase access, and thus initiate transcription (Watkins et al., 2003). The binding of SRC-1 functions co-operatively with ligand binding to PXR to stabilize the AF-2 helix in an active conformation, so that ligand binding is restricted to a single optimal orientation through the restriction of LBD flexibility (Watkins et al., 2003; Orans et al., 2005). This is achieved by three LXXLL motifs present in the co-activator which stabilize the conformation of the AF-2 helix by means of a charge clamp formed through interaction with Lys259 and Glu427 on the surface of PXR (Orans et al., 2005). In contrast, the co-repressors, which include the nuclear receptor co-repressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT), bind to PXR to retain the AF-2 helix in a non-active conformation both in the presence of antagonists and absence of ligand (Stanley et al., 2006). A

Co-regulator type	Co-regulator	PXR	CAR	References/Notes
Co-activators	SRC-1	*	*	(Miao et al., 2006; Stanley et al., 2006; di Masi et al., 2009; Hariparsad et al., 2009; Xie et al., 2009; Azuma et al., 2011)
	NCOA2 (GRIP1, SRC-2)	*	*	
	p/CIP (SRC-3)	*		
	RIP 140	*		
	PGC-1α	*	*	
	PBP	*	*	
	ASC-2		*	
	SMC-1		*	
Co-repressors	SMRT	*		
	NCoR	*	*	
	SHP	*		
	SMILE		*	
	PROX1	*		

Table 1.2.3: Co-activators and co-repressors recruited by PXR and CAR

study investigating SMRT has identified that binding to PXR occurs via the ID2 domain of SMRT (sequence motif: LXXXIXXXI/L), through the interaction of Lys259, Gly270 and Pro423 of the PXR LBD with Arg2347, Lys2348 and Leu2350 of SMRT (Wang et al., 2006). However, charged residues within the PXR LBD also appear to have a core role in the control of PXR activation, with site-directed mutagenesis of Arg410, Asp205, Glu321 and Arg413 indicating an increase in co-repressor binding affinity following mutation to a hydrophobic alanine residue (Wang et al., 2006). This complex interplay between nuclear receptors, ligands, co-activators and co-repressors is therefore responsible for the transcriptional regulation of target genes in response to xenobiotic challenge.

To this point, the PXR-mediated activation cascade for the ligand-induced transcriptional control of target genes has been described. However, recent studies indicate that many of these processes are further regulated by the post-translational modification of PXR (Ding and Staudinger, 2005b; Ding and

Staudinger, 2005a; Lichti-Kaiser et al., 2009a; Pondugula et al., 2009; Blomster et al., 2010; Hu et al., 2010; Biswas et al., 2011; Staudinger et al., 2011; Rana et al., 2012). Staudinger *et al.* (2009) gives a full review of all evidence of post-translational modification in PXR to date, with ubiquitination, SUMOylation, acetylation and phosphorylation having all been reported. However, the evidence base defining the effects and underlying mechanisms of post-translational modification in PXR is currently extremely limited. Phosphorylation is the best characterised modification regulating ligand-induced PXR response. A species-specific response to protein kinase-A (PKA) signalling has been described, with phosphorylation enhancing ligand-induced PXR activation in mice, but repressing PXR activation in rats and humans (Ding and Staudinger, 2005b; Ding and Staudinger, 2005a). Protein kinase-C (PKC), protein phosphatase-1/2a and cyclin dependant kinase-2 (CDK-2) activity have also been implicated in the repression of PXR activity, with the mechanism of PKC-mediated repression being associated with increased NCoR binding affinity whilst concurrently blocking recruitment of SRC-1 (Ding and Staudinger, 2005b; Pondugula et al., 2009). The importance of phosphorylation in the regulation of PXR activity has been further highlighted by a recent systematic site-directed mutagenesis study of predicted phosphorylation sites in PXR, in which heterodimerization, DNA-binding activity, co-repressor recruitment and nuclear translocation have all been found to be dependent on the phosphorylation status of 6 key residues: Ser8, Thr57, Ser208, Ser305, Ser350 and Thr408 (Lichti-Kaiser et al., 2009a). Phosphorylation is therefore an important regulator of PXR activity, and should be carefully considered when administering any substance that activates protein kinases.

PXR is therefore a key regulator of numerous proteins concerned with xenobiotic metabolism, in addition to a number of key physiological functions. Its regulation of target genes is complex, involving numerous accessory proteins in addition to control by post-translational modifications. However, whilst this flexibility is a significant benefit in terms of xenobiotic metabolism, it also increases the potential severity of adverse drug reactions and DDIs, being able to interact with all levels of xenobiotic metabolism, in particular the highly

promiscuous enzyme, CYP3A4, and also potentially interfering with normal physiological function to yield a pathological phenotype (Moore and Kliewer, 2000; Harmsen et al., 2007; Ma et al., 2008b; Harmsen et al., 2009). Full analysis of the potential for drug interaction with PXR is therefore vital to the prediction of DDIs.

1.2.2 CAR

The constitutive androstane receptor (CAR; NR1I3; MB67) was discovered in humans in 1994, followed by mouse in 1997 (Baes et al., 1994; Choi et al., 1997). It is a key regulator of xenobiotic metabolism (Masahiko and Honkakoski, 2000; Maglich et al., 2002; Yamamoto et al., 2003; Dickins, 2004; Moore, 2005; Nakata et al., 2006; Stanley et al., 2006; Tien and Negishi, 2006; Plant, 2007; Tompkins and Wallace, 2007; Monostory and Pascussi, 2008; di Masi et al., 2009; Muntané, 2009; Plant and Aouabdi, 2009). However, CAR also has a significant role in the control of various physiological processes, such as energy homeostasis, lipid metabolism, bilirubin metabolism and haem biosynthesis (Maglich et al., 2002; Maglich et al., 2004; Ding et al., 2006; Roth et al., 2008; di Masi et al., 2009; Finn et al., 2009; Hernandez et al., 2009a; Wada et al., 2009). CAR regulates a wide range of target genes across all phases of xenobiotic metabolism **(Table 1.2.1)**, although CYP2B6 and its non-human orthologues (e.g. Cyp2b10 in mice, CYP2B1/2) are considered to be prototypically induced, and thus of value as a marker of CAR activation (Wei et al., 2000; Wang and Tompkins, 2008). It also displays several traits unusual to the nuclear receptor class of proteins, including an unusual protein structure and constitutive basal activity (Dussault et al., 2002). As a result of this constitutive activity, the novel definition of *inverse agonist* has been derived to describe certain compounds, such as androstanol and androstenol, which actively repress basal CAR activity instead of antagonizing CAR activation in the classical sense (Kenakin, 2004). The constitutive activity of this nuclear receptor also allows target gene transcription to be activated by CAR in the absence of ligand-binding, and thus although it is activated by numerous

endogenous and exogenous chemicals, few of them bind directly to the LBD **(Table 1.2.2)**. The major mechanism of CAR-mediated gene transcription is mediated by the *indirect activation* of CAR by other signalling pathways in response to xenobiotic exposure, with very few chemicals having been identified as direct activators (Moore, 2005; Rencurel et al., 2005; Hernandez et al., 2009a). This constitutive activity is a direct result of the unusual protein structure of this nuclear receptor.

The normal structure of a nuclear receptor consists of 5 domains: 1) N-terminal DNA binding domain, which is highly conserved within the family; 2) hinge region; 3) a highly variable C-terminal domain containing motifs required for transcriptional activation of target genes, including the AF-2 domain, required for ligand-mediated activation, and binding sites for heterodimerization with its binding partner, RXRα; 4) the activation function-1 domain, required for ligand-independent activation; 5) hypervariable F domain (Monostory and Pascussi, 2008). However, CAR is unusual in that it consists of the N-terminal domain, hinge region and C-terminal domain only **(Figure 1.2.3)**(Monostory and Pascussi, 2008). The LBD is smaller than that of PXR at approximately 675Å^3 in humans, and consists of eleven α helices, two 3_{10} helices (H2 and H2'), and three β strands (Suino et al., 2004; Xu et al., 2004). The ligand binding site itself is framed by helices H1-5, H10 and β strands 3-4, meaning that it closely resembles that of the Vitamin D receptor in conformation. However, it is thought that the position of the AF-2 domain within the LBD is responsible for the constitutive activity exhibited by CAR, with disruption of this conformation having been associated with switching CAR to an inactive form, such as observed following treatment with an inverse agonist (Timsit and Negishi, 2007). The conformation of AF-2 is therefore vital to the correct functioning of CAR, and is maintained by the cooperative function of three key structural features (Suino et al., 2004; Xu et al., 2004). Firstly, the rigid structure linking the AF-2 domain to helix 10 is maintained by a unique short Helix-X α-helical turn (Leu336, Ser337, Ala338 and Met339 in humans) together with the single amino acid residue separating Helix-X from the AF-2 domain (M350 human,

T350 mouse), restricting the motion of AF-2 and locking it into its active conformation (Suino et al., 2004; Xu et al., 2004). Secondly, hydrogen bonding

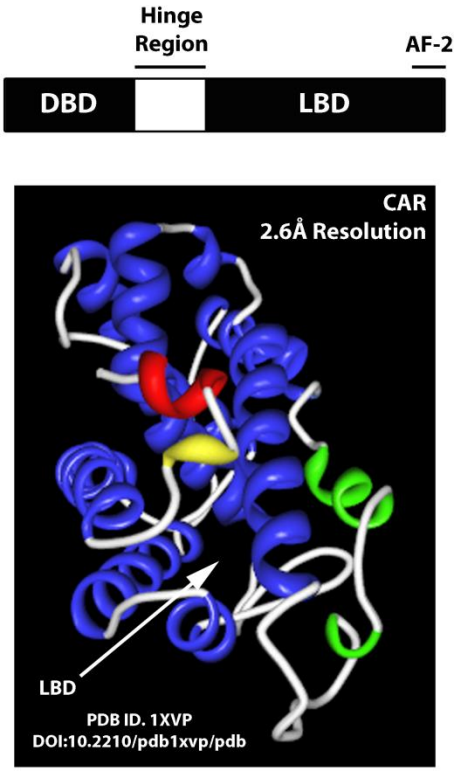


Figure 1.2.3: Representations of hCAR structure

Ribbon depicts domain structure of hCAR, highlighting DNA binding domain (DBD), hinge region, ligand binding domain (LBD) and activation function-2 (AF-2). Crystal structure of hCAR (2.6Å resolution) adapted from PDB ID: 1XVP (Xu et al.*, 2004). Green helices = 3_{10} helices, α2 and α2', yellow helix = Helix-X (H-X), red helix = AF-2. 3D protein structure adapted using RCSB PDB Protein Workshop 4.1.0 (Moreland et al., 2005).*

between the C-terminal carboxylate group of the AF-2 domain and Lys195 from helix 5 in humans (Lys205 in mouse) further stabilizes this structure (Suino et al., 2004; Xu et al., 2004). Finally, the N-terminal domain is stabilized by the H2 helix, which packs tightly against H3, stabilizing the conformation of both H3 and AF-2 (Suino et al., 2004). Importantly, although the conformation of AF-2 is essential for constitutive activity, it is not available for ligand-binding, being protected by the side chains of a highly conserved group of amino acids: Phe161 (also key in constitutive activity), Asn165, Phe234 and Tyr326 (Xu et al., 2004).

Although the structure of CAR results in constitutive activity, this nuclear receptor is not available for activation of downstream target genes in the absence of external stimulus, being normally sequestered in the cytoplasm as part of a complex of CCRP, HSP90 and other accessory proteins (Kobayashi et al., 2003; Kanno et al., 2005b). In response to ligand binding or indirect activation, CAR dissociates from this complex as a result of NLS activation, and is translocated into the nucleus, where it binds to RXR-α prior to activating gene transcription **(Figure 1.2.2)**. The evidence base regarding mechanisms involved in CAR nuclear translocation are better defined than those of PXR, although the molecular trigger is still unclear. It is evident that cellular signalling pathways, and particularly phosphorylation, are an intrinsic component of CAR activation, being involved with its sequestration in the cytoplasm and subsequent nuclear translocation (Zelko et al., 2001) Recent studies have indicated that it is the phosphorylation status of Thr38 in human CAR that is essential to the control of nuclear translocation (Mutoh et al., 2009; Osabe and Negishi, 2011). In response to phosphorylation of Thr38 by PKC, DNA binding is ablated as a result of the disruption of the C-terminal region of the first zinc finger, thus rendering CAR inactive and sequestering it in the cytoplasm as part of the CCRP:HSP90 complex (Mutoh et al., 2009). Dephosphorylation of Thr38 restores CAR activity and promotes nuclear translocation (Mutoh et al., 2009). The essential role of the recruitment of protein phosphatase 2a (PP2A) to the CCRP:HSP90 complex in eliciting nuclear translocation of CAR following phenobarbital treatment has previously been described in mouse hepatocytes (Kawamoto et al., 1999; Yoshinari et al., 2003), and therefore PP2A has been suggested as the mediator of Thr38 dephosphorylation (Mutoh et al., 2009). Another candidate to perform this function is a cell membrane-bound regulatory subunit of protein phosphatase-1β, PPP1R16A, which has also demonstrated the capacity to induce nuclear translocation through interaction with CAR (Sueyoshi et al., 2008). The involvement of this latter protein is further supported by the identification of CAR localization at the cell surface, in addition to the cytoplasm (Koike et al., 2005). However, given the limited evidence for the involvement of both protein phosphatases, further analysis is required to fully elucidate this mechanism.

In terms of regulation, recent studies have implicated the extracellular signal-related kinase (ERK) 1/2 and AMP-activated protein kinase (AMPK) signalling pathways in the control of nuclear translocation. Although it is clear that AMPK has a role in the regulation of nuclear translocation, literature reports of the effect of AMPK activation on nuclear translocation are conflicting (Rencurel et al., 2005; Rencurel et al., 2006; Blättler et al., 2007; Shindo et al., 2007; Kanno et al., 2010a). However, the evidence base supporting the involvement of the growth factor-ERK1/2 pathway is growing, with studies demonstrating that the dephosphorylation of phosphorylated-ERK1/2 is sufficient to induce nuclear translocation (Koike et al., 2007; Mutoh et al., 2009). A recent study has subsequently demonstrated that growth factor-activated phosphorylated-ERK1/2 binds to the XRS located near the C-terminus of CAR, inhibiting dephosphorylation of Thr38, and thus nuclear translocation (Osabe and Negishi, 2011). The XRS is one of three NLS sequences located in rodents, and one of two located in humans. Early studies have identified this region as being associated with nuclear translocation following PB treatment, a hypothesis given some corroboration by the effects of its interaction with phosphorylated-ERK1/2 (Kawamoto et al., 1999; Zelko et al., 2001; Osabe and Negishi, 2011). However, according to later studies, it does not contain a conventional NLS, and has been suggested as a binding site for proteins that mask another NLS, thus inhibiting nuclear translocation prior to ligand activation (Kanno et al., 2005b; Kanno et al., 2007; Xia and Kemper, 2007; Kanno and Inouye, 2008). Further study is therefore needed to examine the precise role of the XRS in nuclear translocation. In terms of the other identified NLS motifs, the first (NLS1) is a basic amino acid sequence, which is well conserved across the *NR1I* family, located within the hinge region of the protein (RRARQARRR; rCAR - amino acids 100-108). However, this region is unable to function as an NLS in hCAR as a result of sequence divergence (Kanno et al., 2007). The second motif (NLS2) is functional in both rodents and humans, and is a non-contiguous group of amino acids located throughout the LBD (amino acids 111-320)(Kanno et al., 2005b; Kanno et al., 2007). This is the NLS potentially masked by proteins bound to the XRS. Once the nuclear receptor is ligand-activated and released from the cytoplasmic CAR retention complex, active CAR is translocated into the nucleus

via an importin/Ran-GTP mediated process in which IPO13 binds to NLS2 for nuclear translocation (Kanno et al., 2010b).

Once in the nucleus and bound to RXR-α, the heterodimer binds to recognition sequences in the target genes to initiate transcription. CAR preferentially recognises 3 binding motifs: DR-4 (e.g. AGTTCAnnnnAGTTCA), DR-5 (e.g. AGTTCAnnnnnAGTTCA) and ER-8 (e.g. TGAACTnnnnnnnnAGTTCA), although it can also bind other motifs, such as ER-5 and ER-10, with lower affinity (Frank et al., 2003; di Masi et al., 2009). There is also potential for CAR-mediated transcriptional activation as a result of binding of a CAR monomer to DR-4 motifs, and particularly the sequence AGAGTTCA, although the frequency and efficiency of this mechanism has not been assessed (Frank et al., 2003). However, it does appear that the preference for monomeric binding is stronger in humans than mice (Frank et al., 2003). Co-factors are recruited following CAR binding to target gene promoters to modulate gene expression **(Table 1.2.3)**.

CAR-mediated regulation of xenobiotic metabolism is therefore highly complex, relying on structural changes, post-translational modification and interaction with other signalling pathways to modulate target gene transcription. However, the numerous physiological roles regulated by CAR make this nuclear receptor particularly susceptible to influence from endogenous systems. A key example is the impact of energy state on drug pharmacokinetics and pharmacodynamics as a result of the modulation of genes associated with xenobiotic metabolism (Hernandez et al., 2009a). The consideration of functional cross-talk between CAR-regulated systems is therefore essential to the analysis of potential CAR-mediated DDIs. CAR is also subject to circadian control as a result of its regulation by the PAR-domain basic leucine zipper transcription factors albumin D-box binding protein (DBP), thyrotroph embryonic factor (TEF) and hepatic leukaemia factor (HLF) (Gachon et al., 2006). It may therefore be necessary to consider the potential for DDIs as a result of variability in drug pharmacokinetics caused by CAR-mediated chronotherapeutic effects. In summary, although CAR is less promiscuous than PXR, the analysis of CAR-

mediated DDIs is complicated by functional cross-talk with endogenous systems, as well as elements involved with its own regulation.

1.2.3 CONTROL OF KEY P450S: NUCLEAR RECEPTOR CROSS-TALK

Xenobiotic metabolism encompasses a highly complex and robustly controlled network of interacting proteins essential to protect the body from xenobiotic challenge. This is reflected in the complexity of its regulatory network, in which multiple nuclear receptors and transcription factors interact to provide robust, fine control of this system, a phenomenon commonly known as receptor cross-talk (Pascussi et al., 2008). In part, cross-talk describes the redundancy inherent in the system, with multiple receptors capable of being activated by the same ligand or signalling pathway, recruiting the same co-activators/co-repressors, or interacting with the same recognition sequences in target gene promoters. However, it is this cross-talk which is responsible for the robust response against xenobiotic challenge demonstrated by the system, as well as the control of interactions between endogenous and exogenous pathways. It is imperative that potential cross-talk between regulatory elements should be assessed when considering the development of DDIs, with the analysis of one receptor only providing an unrealistic representation of the effects in an inherently complex system. A full review of the regulatory control of various P450s can be found in Fraser *et al.* (2012). However, only regulatory cross-talk relevant to the expression of CYP2B6 and CYP3A4, which are prototypically induced by hCAR and hPXR respectively, will be considered in this introduction.

1.2.3.1 CYP2B6

Although the role of CYP2B6 has been considered minor, accounting for only 6% of total hepatic microsomal protein expression, it is responsible for the metabolism of a wide variety of drugs, including cyclophosphamide, valproic acid, ketamine, methadone, bupropion, nicotine, and methylenedioxymethamphetamine (MDMA) (Wang and Tompkins, 2008; Benet et al., 2010a; Lo et al., 2010). Activated hCAR is a key regulator of this gene,

binding to a DR-4 motif located in the phenobarbital responsive element module (PBREM) and an imperfect DR-4 located in the distal xenobiotic response element (XREM) of the promoter (Monostory and Pascussi, 2008; Wang and Tompkins, 2008). However, hPXR also demonstrates an affinity for these DR-4 binding sites, although with a lower activity than hCAR, and thus has been implicated in the regulation of CYP2B6 (Sueyoshi et al., 1999; Goodwin et al., 2001; Wang et al., 2003; Wang and Tompkins, 2008). A recent study has identified hPXR-mediated induction of *CYP2B6* in primary human hepatocytes in response to treatment with oltipraz (Piton et al., 2010). Interestingly, hPXR has also been linked to an increase in *CYP2B6* expression observed in populations possessing the -82 T→C single nucleotide polymorphism (SNP) in the promoter region of CYP2B6 (Li et al., 2010). This SNP introduces a CCAAT/enhancer binding protein-α (CEBPα) site into the promoter region, with which PBREM-bound hPXR is proposed to interact, synergistically inducing expression.

Cross-talk between CAR and PXR are therefore of importance for the control of *CYP2B6* expression. However, numerous other receptors and liver-enriched transcription factors are implicated in the regulation of this protein, with the proposed regulatory network being shown in **Figure 1.2.4**. Studies have indicated that the okadaic acid response element ($OARE_{KI}$) is required for maximal activation of PBREM-mediated gene transcription (Inoue and Negishi, 2008). This region contains a HNF4α DR-1 binding site, a CACCC motif, and an E-box motif to which early growth response protein-1 (EGR1) binds. Co-operative functioning of these motifs is essential to achieving maximal CAR-mediated *CYP2B6* expression, with EGR1 binding to the E-box motif inducing DNA looping and facilitating the synergistic interaction of HNF4α bound to the $OARE_{KI}$ and active CAR bound to the PBREM (Inoue and Negishi, 2009). The position at which HNF4α is bound to the *CYP2B6* promoter is also of importance when defining its regulatory interactions (Benet et al., 2010a). When bound to the $OARE_{KI}$ binding site, HNF4α interacts as described above. However, when bound to a distal DR-1 HNF4α binding element located at -1642bp upstream of the *CYP2B6* gene, the favoured interaction is between HNF4α and C/EBPα

bound to an element located at -1597bp, leading to CAR-independent CYP2B6 upregulation (Benet et al., 2010a).

It is therefore clear that cross-talk between CAR, EGR1, HNF4α and C/EBPα is required for maximal induction of *CYP2B6*. However, studies to date have examined a very limited set of potential interactors with respect to regulatory cross-talk. Importantly, there has been no analysis at present regarding the interaction between the XREM and other regulatory elements to support expression of this gene. The interaction between the pathway characterised above and other transcriptional regulators has not been assessed. No specific

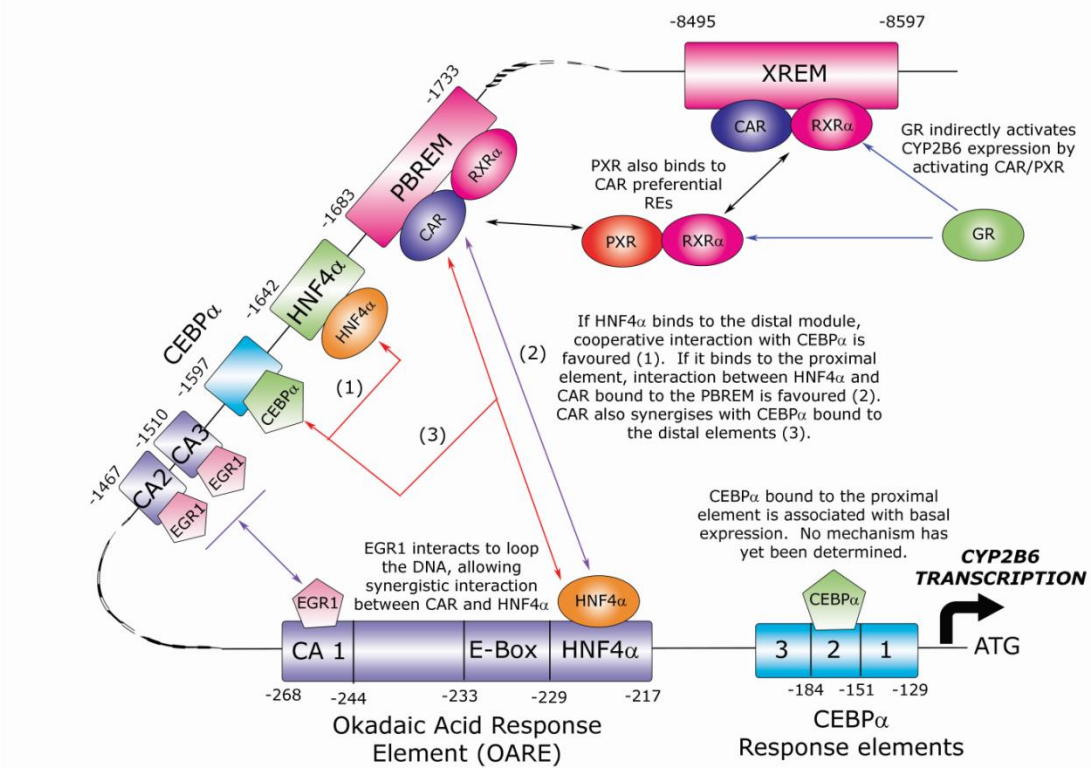


Figure 1.2.4: Regulatory network controlling CYP2B6 expression.

Source: Adapted from Fraser et al. (2012)

interactions with PXR have been analysed at present, although there is evidence for PXR-mediated gene regulation. The glucocorticoid receptor has been found to indirectly mediate CYP2B6 expression through the induction of hCAR and hPXR, although in rodents it is able to directly mediate the expression of CYP2B6 orthologues (Meehan et al., 1988; Schuetz et al., 2000; Wang and

Tompkins, 2008). The oestrogen receptor-α (ERα) has also been implicated in the induction of CYP2B6, although currently the mechanism underlying this is unclear. One study indicates induction mediated by ERα interaction with an oestrogen responsive element located at -1669 to−1657bp upstream of the *CYP2B6* gene (Lo et al., 2010), whilst another favours ERα interaction with an AP-1 site located upstream of the *CYP2B6* gene, with synergistic action between this motif and the CAR-bound PBREM in conditions of high oestradiol concentrations, such as are observed in pregnancy (Koh et al., 2012). Although significant advances have been made in our understanding of *CYP2B6* transcriptional regulation, the complexity of the regulatory network means that further analysis is required. However, the key role of CAR in *CYP2B6* regulation is well established, together with the importance of regulatory cross-talk to modulate gene expression.

1.2.3.2 CYP3A4

The importance of CYP3A4 in xenobiotic metabolism has been discussed in *Section 1.1.2*. In terms of regulation, *CYP3A4*, and its mouse orthologue *Cyp3a11*, are both prototypically induced by PXR following drug activation. For maximal induction of *CYP3A4*, synergistic interaction of 3 regions is required (Liu et al., 2008): 1) An ER-6 PXR response element (PXRE) at -172bp upstream of the start codon in the proximal promoter (Barwick et al., 1996; Goodwin et al., 1999; Quattrochi and Guzelian, 2001); 2) the dNR1 imperfect DR-3 binding motif and dNR3 sites located in the distal XREM enhancer region at -7800bp (Goodwin et al., 1999); and 3) the constitutive liver enhancer module of *CYP3A4* (CLEM4) located at -11.4 kbp, which contains multiple enhancer binding sites, including hepatocyte nuclear factor-1 (HNF1), HNF4α and AP-1, as well as a perfect ER-6 PXR binding motif which appears specific to a PXR homodimer complex which is as yet unidentified *in vivo* (Matsumura et al., 2004; Liu et al., 2008). It is the binding motifs in the PXRE and XREM that are essential to *CYP3A4* expression, with mutation in both reducing rifampicin-induced *CYP3A4* expression by 85% (Goodwin et al., 1999). CLEM4 is also associated with the constitutive expression of this gene, although it is not essential, with disruption

of the CLEM4 ER-6 motif resulting in no change in basal expression, whereas disruption of the XREM DR-3 motif causes a reduction in expression (Matsumura et al., 2004; Liu et al., 2008). All binding sites described above are also able to bind hCAR, thus implicating CAR in *CYP3A4* regulation (Goodwin et al., 2002; Urquhart et al., 2007). However, studies have shown that hCAR binding of the PXRE motif alone is not sufficient to induce transcription of this gene, suggesting that hCAR acts co-ordinately with hPXR (Goodwin et al., 2002; Urquhart et al., 2007).

The role of interacting transcription factors in *CYP3A4* regulation is not as well defined as those involved in the regulation of *CYP2B6*. The role of HNF4α has been the best defined, being a key regulator of PXR- and CAR-induced *CYP3A4* transcriptional regulation, with its role being dependent on to which of its recognition sequences it binds (Tirona et al., 2003; Liu et al., 2008). When bound to the DR1 recognition site located at -7783 bp in the distal XREM, HNF4α synergistically interacts with PXR/CAR to induce promoter activity in transactivation assays performed in HepG2, Caco-2 and HeLa cell lines (Tirona et al., 2003). However, when bound to the two response elements located downstream of the PXRE located in CLEM4, HNF4α represses PXR/CAR-mediated transactivation by disrupting nuclear receptor DNA binding (Liu et al., 2008). Interestingly, C/EBPα has also been implicated in *CYP3A4* regulation, with disruption of a putative recognition site located at -132 bp repressing basal expression by 60%, as well as maximal expression induced by rifampicin, phenobarbital and metyrapone (Bombail et al., 2004; Martínez-Jimínez et al., 2007). Two further C/EBPα binding motifs associated with *CYP3A4* activation have also been identified in the *CYP3A4* promoter at -1402 and -1668 bp. However, the exact role of C/EBPα in regulation of this gene has not yet been defined, although a synergistic interaction between C/EBPα and HNF3γ (FOXA3; bound to a recognition site at -1730 bp) which results in increased transcription as a result of chromatin remodelling has been described (Rodríguez-Antona et al., 2003; Bombail et al., 2004). A putative Sp1 site (-104 to -97 bp) has also been suggested to be important for *CYP3A4* expression, with disruption of this site being responsible for a 50% reduction in promoter

activity (Bombail et al., 2004). Other regulators reported to be associated with *CYP3A4* regulation include STAT1, HNF1 and HNF3β (FOXA2), although the mechanisms underlying their role have yet to be determined (Martínez-Jimínez et al., 2007).

In addition to those entities that directly interact with the *CYP3A4* promoter, there are also a number that regulate gene expression through interaction with PXR and CAR. For instance, the glucocorticoid receptor is reported to modulate PXR expression, leading to induction of *CYP3A4*, rather than acting through a putative glucocorticoid response element identified in the *CYP3A4* gene (Khan et al., 2009; Zimmermann et al., 2009) Hypoxia inducible factor-1α (HIF-1α) represses *CYP3A4* in the HepaRG cell line in response to oxidative stress by an indirect mechanism, such as modulation of regulatory proteins including PXR and CAR (Legendre et al., 2009). However, the mechanism underlying this repression has not yet been characterised. A number of transcription factors are also able to bind the ER-6 and DR-3 motifs preferentially targeted by hPXR, and thus to regulate *CYP3A4* through competition or synergism with hPXR (Matsumura et al., 2004; Istrate et al., 2010). Thyroid receptor α1 (TRα1) competes with hPXR to bind these elements, repressing basal and xenobiotic-induced expression (Istrate et al., 2010). This competition has been implicated in the basal regulation of *CYP3A4* activity, with the TRα1:PXR ratio being correlated with basal gene expression. It has also been reported that the vitamin D receptor (VDR) can compete for ER-6, DR-3 and DR-4 motifs, inducing *CYP3A4* expression with a lower activity than PXR (Drocourt et al., 2002; Khan et al., 2009). However, VDR has also been shown to synergize with hPXR in the control of this gene, through VDR binding to response elements in CLEM4 (Pavek et al., 2010). Finally, PXR-mediated *CYP3A4* expression will also be modulated by the interaction of endogenous signalling pathways with this nuclear receptor *(See Chapter 1.2.1)*. One example is the regulation of *CYP3A4* as part of the cell cycle as a result of PXR phosphorylation by cyclin-dependent kinase 2 (Lin et al., 2008).

CYP3A4 regulation is therefore far more complex than that of *CYP2B6*, probably reflecting the greater importance of this enzyme in defence against xenobiotic challenge. Unfortunately the complexity of the regulatory network hinders specific analysis of interactions between regulatory elements. Systematic analysis of the interaction between regulatory elements is therefore required to provide a more coherent mechanistic understanding of the key elements of *CYP3A4* regulation, and thus specific elements that may increase the risk of DDIs.

In conclusion, examining the regulation of *CYP2B6 and CYP3A4* has demonstrated the key role of CAR and PXR in the transcriptional regulation of these genes. Cross-talk between various regulatory elements involved with transcriptional control is obviously a highly complex system, encompassing elements of endogenous systems as well as those specifically related to xenobiotic metabolism. Whilst recent studies have begun to elucidate functional connections between binding elements in promoter regions, in addition to regulatory networks that indirectly influence gene expression, there is still significant research required before gene transcription can be fully understood. The importance of assessing the interplay between xenobiotic metabolism and endogenous systems is also highlighted by the capability of endogenous molecules to regulate xenobiotic metabolism. However, care must be taken when dissecting receptor-specific effects from experimental models in which regulatory cross-talk is inevitable, both *in vitro* and *in vivo*. Selecting models that reduce the risk of generating erroneous data in pre-clinical molecular pharmacology studies is therefore an essential step in methodological design.

1.3 PROJECT AIMS

The ability to correctly predict DDIs from preclinical studies is an essential part of drug design. With a growing understanding of the role of nuclear receptors in the control of xenobiotic metabolism has come the recognition that drugs that interact with these nuclear receptors could cause significant adverse

effects, including altered drug metabolism and potential toxicity. Any ligand interacting with a nuclear receptor controlling xenobiotic metabolism, such as PXR and CAR, is therefore at risk of causing DDIs. Characterising novel therapeutics with respect to their potential to activate PXR and CAR is therefore an essential step towards the identification of DDIs. However, the majority of experimental approaches are unable to effectively analyse the effects of activation in a meaningful manner, because they are unable to model and dissect mechanisms underlying the regulatory cross-talk between these two receptors *(See Chapters 3 & 4)*.

This study has been designed to allow regulatory cross-talk between PXR and CAR in response to xenobiotic challenge to be assessed. Initially, a panel of commonly used anti-neoplastic agents shall be analysed in a wild-type C57BL/6J mouse model to identify drugs with the potential to activate PXR or CAR, with up-regulation of Cyp3a11 and Cyp2b10 being used as a marker. Those drugs that demonstrate probable interaction with these nuclear receptors will then be subjected to a systematic analysis of PXR and CAR activation potential using a novel panel of transgenic mice which allow cross-talk between these two receptors to be fully assessed (Scheer et al., 2008; Ross et al., 2010; Scheer et al., 2010). These studies will investigate the role of PXR and CAR in modulation of P450 protein expression, enzyme activity and drug pharmacokinetics, providing both regulatory and phenotypic data. A novel reporter model will also be employed to investigate species-specific variations in CYP2B6 expression (*model unpublished*). This data will provide a solid basis for future studies, in which the potential for DDIs following administration of these drugs can be assessed by means of co-administration studies.

CHAPTER 2

MATERIALS AND METHODS

2.1 General

2.1.1 Materials and Chemicals

All chemicals unless otherwise stated were purchased from Sigma-Aldrich (UK). All cell culture reagents were purchased from Life Technologies, Paisley, UK. All anti-cancer drugs for *in vivo* studies, including Femara (letrozole, Novartis) and Arimidex (anastrozole, Astra-Zeneca) were obtained via Ninewells hospital pharmacy (Dundee, UK). Euthatal anaesthetic was purchased from Meriel Animal Health (Harlow, Essex, UK). Cell+ TC flasks, 75 cm^2 were purchased from Sarstedt (Leicester, UK). Phosphate buffered saline (PBS) was purchased from Oxoid (Cambridge, UK). RQ1 RNase-free DNase, random primers, dNTPs and nuclease-free water were purchased from Promega (Southampton, UK). All other cDNA and TaqMan® real-time PCR reagents were purchased from Life Technologies (Paisley, UK). Bio-Rad protein assay, XT MOPS running buffer and all elements of the Criterion blotting system, including pre-cast gels, were purchased from Bio-Rad Laboratories Ltd (Hemel Hempstead, UK). Polylysine microscope slides, Whatman® Protran® nitrocellulose membrane, Whatman® filter paper and methanol for Western blotting were purchased from VWR International Ltd (Lutterworth, UK). Full-range rainbow molecular weight markers were purchased from GE Healthcare (Chalfont St. Giles, UK). Secondary antibodies were obtained from Dako UK Ltd (Ely, UK). Immobilon chemiluminescent horseradish peroxidase substrate was purchased from Millipore (Watford, UK). 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-hydroxy-4-trifluoromethylcoumarin (HFC), 7-benzyloxyquinoline (BQ) and 7-hydroxyquinoline (HQ) were purchased from Cypex Ltd. (Dundee, UK). 7-methoxy-4-trifluoromethylcoumarin (MFC) and 7-ethoxy-4-trifluoromethylcoumarin (EFC) were purchased from BD Gentest (Cowley, UK). NADPH was obtained from Melford Laboratories (Ipswich, UK). Letrozole and anastrozole powders for standard curves were purchased from Tecoland (New Jersey, USA). HPLC grade acetonitrile, methanol and dichloromethane were obtained from Rathburn Chemicals Ltd (Walkerburn, Scotland). MS grade

water, methanol and diethylether were purchased from Fisher Scientific (Loughborough, UK). Columns for HPLC and UPLC-MS-MS analysis were purchased from Acquity (U.S.A.). Histoclear II was obtained from National Diagnotics U.S.A. (Georgia, U.S.A.). Nuclear fast red dye was purchased from Vector Laboratories Ltd (Peterborough, UK).

2.1.2 Statistical Analysis

Statistical analysis was performed using SigmaPlot 12.0 (Systat Software, Inc.). One way analysis of variance (ANOVA) using the Holm-Sidak post-hoc test were performed. *P* values are represented by asterisks, with * representing $p \leq 0.05$, ** representing $p \leq 0.01$ and *** representing $p \leq 0.001$.

2.2 Animals

2.2.1 Animal Maintenance

All animal procedures were carried out in accordance with the Animals (Scientific Procedures) Act (1986) and after local ethical review. Animals were maintained in standard animal house conditions with *ad libitum* access to standard rodent diet and water, and a 12 hour light/dark cycle.

2.2.2 Animal Models

All mouse models used in these studies were based on a C57BL/6J genetic background. Wild type mice were obtained from colonies bred in-house. Information regarding supply and construction of the transgenic mouse lines used are detailed in **Table 4.2.1**. All models were bred and genotyped in-house following initial supply.

2.2.3 *IN VIVO* STUDIES

In vivo methods for initial drug screening and transgenic studies are given in *Section 3.2.1* and *4.2.1* respectively.

2.2.3.1 E0771 SYNGENEIC MODEL PILOT STUDY

E0771 murine breast adenocarcinoma cell culture

The E0771 cell line, obtained from Dr Fengzhi Li (Roswell Park Cancer Institute, Buffalo), is a murine metastatic breast adenocarcinoma cell line derived from C57Bl/6J mice for use as a syngeneic tumour model (DeGraw et al., 1993; Sirotnak et al., 1993; Mihich and Ehrke, 2000; Ewens et al., 2005; Ewens et al., 2006; Gu et al., 2009; Stagg et al., 2010; Young et al., 2010; Gu et al., 2011). Using aseptic techniques, E0771 cells were maintained in Cell+ TC flasks, 75 cm^2 (Sarstedt, UK) in an incubator at 37°C in 5% CO_2 using RPMI 1640 (+25mM HEPES, +GlutaMAX™) supplemented with 10% foetal bovine serum (E.U. approved, South American origin) and penicillin-streptomycin (100x). Cells were split 1:50 twice weekly when they had reached approximately 70-80% confluency. The cell layer was washed twice with 10 ml sterile PBS, then covered with 1 ml TrypLE™ Express and incubated at 37°C for 3 minutes to allow cell dissociation. 9 ml growth medium was added to the flask and cells were finally dissociated from the flask using gentle pipetting. The cell suspension was removed to a 15ml falcon tube and centrifuged at 1,000 rpm for 3 minutes in a benchtop centrifuge. The supernatant was removed by aspiration, ensuring the cell pellet was not disturbed. The cell pellet was resuspended in 12.5ml growth medium, with 250µl being transferred into 25ml growth medium in a fresh flask, rocking to evenly distribute cells. Medium was changed 3 days after seeding, and daily thereafter.

Prior to injection into mice, cells were tested in house for mycoplasma infection, and sent to Charles River Laboratories (Margate, UK) for mouse essential panel

pathogen analysis to ensure that they were free from common mouse and cell based pathogens. To produce the cell suspension for injection, when the cells reached 80-90% confluency they were dissociated from the flask using the protocol above. However, a cell count was performed on a sample of cell suspension before continuing to the centrifugation step. After the supernatant was removed from the cell pellet, the pellet was washed by resuspending in 10ml PBS followed by centrifugation at 95 *x g* for 3 minutes. The supernatant was aspirated and the cells were washed once more. The supernatant was aspirated from the cell pellet and the cells were resuspended at 2.5 x 10^6 and 1.25 x 10^6 cells/ml in Hanks' balanced salt solution (HBSS; Life Technologies, UK), then transferred on ice ready for injection.

E0771 syngeneic tumour model

Under Euthatal anaesthesia (Harlow, UK), female C57Bl/6J wild type (n=5) and HuPXR/HuCAR transgenic mice (n=4) were injected *s.c.* into both flanks with either 2.5 x 10^5 (right hand side) or 1.25 x 10^5 (left hand side) E0771 cells suspended in 100 μl HBSS (Day 0). Mice were weighed 5 times a week to monitor potential distress, and checked for tumours 3 times a week. Once large enough, tumours were measured in 3 dimensions, 3 times a week, and a geometric mean diameter (GMD) calculated using the formula $\sqrt[3]{lxbxh}$. Mice were euthanized by rising CO_2 once GMD ≥ 12.5mm, or if the mouse showed signs of distress or other pathology, such as tumour ulceration.

Following euthanasia, blood was harvested by cardiac puncture into heparinized tubes which were subsequently centrifuged at 16,000 *x g* for 10 minutes. Plasma supernatant was removed to a clean eppendorf which was snap frozen in liquid N_2 and stored at -70˚C prior to analysis. Each mouse was assessed for internal metastases by eye, with any secondary tumours being harvested. Liver was weighed and bisected, with one half being transferred to fixative, and the other half being transferred to a bijou, snap frozen in liquid N_2 and stored at -70˚C prior to analysis. Both lungs were harvested into fixative. Primary and secondary tumours were harvested and bisected to check for

necrosis. Where large enough, 1/3 tumour was transferred to fixative with the remaining 2/3 being snap frozen in liquid N_2 and stored at -70˚C prior to analysis. Where the tumour was too small, the entire tumour was fixed. Where tumours had invaded skin or muscle, a portion of surrounding tissue was also harvested to fixative. All fixative samples were fixed in GURR (formal saline). The GURR (formal saline) samples were left at 4˚C overnight, then rinsed twice in PBS and transferred to 70% ethanol for storage at 4˚C prior to wax embedding

2.3 Cytochrome P450 analysis

2.3.1 Gene Expression Analysis

2.3.1.1 RNA Extraction and Quantitation

RNA was extracted using the "TRIzol® reagent with Purelink™ Micro-to-Midi system" protocol as given by the manufacturers (Life Technologies, UK). RNA concentration was assayed using a UV spectrophotometer measuring absorbance at 240nm. Each sample was diluted 1:200 in nuclease-free water (Promega, UK) and was read together with a nuclease-free water blank. Purity was assessed by measuring absorbance at 260nm. After quantitation, dilutions were produced for each sample at 100ng/µl in nuclease-free water in preparation for cDNA synthesis.

2.3.1.2 cDNA synthesis

Prior to cDNA synthesis, the RNA was DNase treated. In a final reaction volume of 10µl, 600ng of diluted RNA (100ng/µl) was mixed with RT buffer (1x; Life Technologies, UK), RQ1 RNase-free DNase (Promega, UK) and DEPC treated water (Life Technologies, UK). The reaction was heated to 37°C for 10 minutes,

after which 1µl RQ1 Stop buffer (Promega, UK) was added to the reaction, mixed and incubated at 65°C for 5 minutes to stop the reaction.

To start cDNA synthesis, 150ng random hexamers (Promega, UK) were added to the reaction, which was then heated to 70°C for 10 minutes before being chilled on ice and the contents being spun down. A master mix was produced, with each reaction receiving 8 µl in total, containing RT buffer (1x; Life Technologies, UK), DTT (100nmol; Life Technologies, UK), dNTPs (10nmol; Promega, UK) and DEPC treated water. This was incubated at 25°C for 5 minutes before the addition of 100U Superscript reverse transcriptase (Life Technologies, UK). The reaction was then heated in a thermal cycler using the following settings: 25°C for 10 minutes → 42°C for 50 minutes → 70°C for 10 minutes → 4°C ∞. The samples were diluted 1:10 to obtain a 3ng/µl final concentration, based on input RNA and stored at -20°C.

2.3.1.3 TaqMan Real-Time PCR

Singleplex TaqMan® real-time PCR gene expression assays (Life Technologies, UK) were run in a 96 well plate format using a Life Technologies, UK 7700 Real-Time PCR system. Samples were run in triplicate using the following reaction mix: 2.5 µl cDNA, TaqMan® Universal PCR Master Mix (1x), TaqMan® gene expression assay (1x) and nuclease-free water to a final volume of 12.5µl. The reactions were performed according to the manufacturer's instructions. Mouse *cyp3a11* (Mm00731567_m1), *cyp2b10* (Mm01972453_s1) and *cyp2c29* (Mm00725580_s1) genes were analysed alongside the eukaryotic 18s ribosomal RNA housekeeping gene (Hs99999901_s1). The data was analysed to give a fold difference relative to saline control using the comparative Ct (ΔΔCt) method as described by the manufacturer.

2.3.2 PROTEIN ANALYSIS

2.3.2.1 ISOLATION OF MICROSOMAL FRACTION

Snap-frozen liver portions (1/3) were defrosted on ice in 2ml KCl-Phosphate buffer ($KHPO_4$ [0.01M, pH7.4], di-sodium EDTA [0.1mM], KCl [1.15%]) prior to scissor mincing and mechanical homogenization. The homogenate was centrifuged at 11,500 *x g* in a benchtop centrifuge at 4°C for 20 minutes. The supernatant, avoiding the fatty upper layer, was transferred to ultracentrifuge tubes (maintained on ice) and balanced prior to centrifugation at 100,000 x g at 4°C for 80 minutes. The supernatant (cytosolic fraction) was transferred to a fresh eppendorf tube and stored at -70°C. The pellet (microsomal fraction) was resuspended in 200-800µl KCl-Phosphate/Sucrose storage buffer ($KHPO_4$ [0.01M, pH7.4], di-sodium EDTA [0.1mM], KCl [1.15%], 0.25M sucrose) by hand homogenization. The homogenate was divided into 100µl aliquots and maintained on ice prior to storage at -70°C.

2.3.2.2 PROTEIN CONCENTRATION DETERMINATION

Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories Ltd, UK). A standard curve was produced by spiking 1ml Bio-Rad protein assay reagent (1x in PBS) with 0, 3, 6, 9 or 12 µg bovine serum albumin protein standard (1 mg/ml). Samples were assayed in duplicate, using a 1:1000 dilution in 1x Bio-Rad protein assay reagent to enable direct read-out of concentration. Samples and standards were produced and mixed in visible cuvettes. Absorbance was measured using a visible light spectrophotometer at 595nm, and protein concentration calculated from the standard curve.

2.3.2.3 WESTERN BLOTTING

Prior to western blotting, samples were mixed with NuPAGE® LDS Sample buffer (1x; Life Technologies, UK) and β-mercaptoethanol (10%), to give a final protein concentration of 2 mg/ml. They were then denatured by heating to 100°C for 10 minutes.

Proteins were separated by electophoresis using the Bio-Rad Criterion™ pre-cast SDS-PAGE gel system (Bio-Rad Laboratories Ltd, UK). Criterion™ XT Bis-Tris pre-cast gels (26 wells, 4-12% [Cyp2c only]/10% [all other genes]; Bio-Rad, UK) were set up as per the manufacturer's instructions using XT MOPS running buffer (1x; Bio-Rad, UK). Gels were loaded with protein marker (5μl, GE Healthcare, UK), positive control (5μg dexamethasone-induced male wild-type liver microsomes [Cyp3a/POR]/ 10μg phenobarbital-induced male wild-type liver microsomes [all other genes]), and samples (20μg). All wells were loaded with a constant volume (10 μl), with those wells containing less than this volume being filled with NuPAGE® LDS Sample buffer (1x; Life Technologies, UK). Electrophoresis was started at a voltage of 100V for 20 minutes to allow better stacking of proteins, before being increased to 200V until the 31kDa marker had just run off the bottom of the gel.

The proteins were transferred to nitrocellulose membrane (VWR International, UK) using the Criterion™ blotter. Nitrocellulose membrane (1 per gel) and Whatman filter paper (4 per gel; VWR International, UK) were cut to the same dimensions as the blotting sponge pads (2 per gel) (13cm x 9cm). These were pre-soaked in transfer buffer (Tris base [16mM], glycine [120mM], methanol [20% v/v; VWR International, UK]) prior to the end of SDS-PAGE electrophoresis. Upon completion, the SDS-PAGE gel was removed from the cassette and the stacking gel was removed. A sandwich was then produced as follows: One sponge pad was placed on the cathode side of the cassette and covered with 2 pieces of Whatman paper. The gel was briefly equilibrated in transfer buffer before being placed on the filter paper and carefully covered with the nitrocellulose membrane. A further 2 sheets of Whatman paper were

placed on top of the membrane, and the bubbles were carefully removed using a roller. The final sponge pad was placed on top of the sandwich and the cassette was closed, ensuring that the layers do not move. The cassette was transferred to the Criterion™ blotter, together with a cooling block, and was then filled with transfer buffer. The tank was placed in a tray full of ice to ensure that the module did not overheat and damage the proteins. Proteins were transferred to the nitrocellulose membrane using a voltage of 100V for 90 minutes. Equal transfer and loading was confirmed following electrophoresis using Ponceau S stain. The stain was rinsed from the membrane firstly using distilled water, and then Tris buffered saline-Tween (NaCl [154mM], Tris base [50mM], Tween [0.1% v/v]; TBST). The membrane was blocked overnight in milk:TBST solution (10% w/v).

Membranes were incubated in polyclonal antisera diluted in milk:TBST solution (5% w/v) for 4 hours (see Table 2.3.1 for dilutions; all antibodies generated in house). They were then washed in TBST for 4x 5 minutes on a rocker set at high speed, and incubated for 1 hour in the relevant secondary polyclonal, horseradish-peroxidase linked antibody (1:10,000 in TBST) (Table 2.3.1; Dako UK Ltd., Ely, UK), before washing as previously described. The blots were visualized on a Fujifilm LAS-3000 mini-imaging system (Fujifilm UK Ltd., Bedfordshire, UK) using Immobilon chemiluminescent horseradish peroxidase substrate (Millipore, Watford, UK). Densitometric analysis was performed using MultiGauge version 2.2 software (Fujifilm UK Ltd.).

Antibody	Raised against	Dilution	Secondary Antibody
Cyp1a	Mouse	1:10000	Goat anti-mouse
Cyp2a	Sheep	1:5000	Goat anti-sheep
Cyp2b	Rabbit	1:2000	Goat anti-rabbit
Cyp2c		1:5000	
Cyp3a		1:2000	
POR		1:2000	

Table 2.3.1: Antibodies used for western blotting

2.3.2.4 Cytochrome P450 Activity Determination

Fluorigenic Cytochrome P450 activity assays were performed in 96 well white assay plates using a final reaction volume per well of 150µl. Each incubation was performed in triplicate in 50mM Hepes pH7.4, 30mM $MgCl_2$ and contained 30µg liver microsomal protein together with one probe substrate at the concentration given in T**able 2.3.2**. The incubation mix was then warmed to 37°C before the reaction was started by the addition of 0.67mM NADPH (Melford Laboratories, Ipswich, UK). The reaction was measured in real time for 3 minutes using the excitation and emission wavelengths given in table 2.3.2 on a Fluroskan Ascent FL plate reading fluorimeter (Labsystems, UK). Turnover rates were calculated using standard curves generated using metabolite standards (see table 2.3.2).

Substrate (S)	[S] /μM	Enzyme detected	Metabolite	$\lambda_{excitation}$ /nm	$\lambda_{emission}$ /nm
7-benzyloxy-4-trifluoromethyl-coumarin (BFC)	30	***CYP3A4*** ***CYP1A2*** *CYP2C19* *CYP1B1* *CYP1A1*	7-hydroxy-4-trifluoromethyl-coumarin (HFC)	405	530
7-ethoxy-4-trifluoromethyl-coumarin (EFC)	40	CYP2B6 CYP2C19 CYP2E1			
7-methoxy-4-trifluoromethyl-coumarin (MFC)	120	CYP2C9 CYP2B6 CYP2E1			
7-benzyloxy-quinoline (BQ)	40	***CYP3A4*** *CYP1A1/2*	7-hydroxy-quinoline		
Methoxyresorufin (MR)	0.001	CYP1A2	Resorufin	530	584
Ethoxyresorufin (ER)		CYP1A1/2 CYP1B1			
Benzoxyresorufin (BR)		CYP2B6			

Table 2.3.2: Fluorigenic activity assay probe data (human/rat only)

Isoform = Major isoforms. *Isoform = Minor isoform. Isoforms listed in order of contribution to overall activity.*

2.3.3 Pharmacokinetic Analysis

2.3.3.1 Sample Preparation

All samples were extracted using a solvent extraction method with an associated standard curve to account for differences in extraction efficiency. Letrozole and anastrozole powders (Tecoland, New Jersey, USA) were both reconstituted in acetonitrile (Rathburn Chemicals Ltd, Scotland). For each standard curve, the relevant drug was spiked into untreated whole mouse

blood:heparin (15IU/ml; 50:50 v/v) to give the following concentrations in a final volume of 20μl: 0, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 μg/ml.

To both samples and standards, 100μl internal standard was added (Letrozole – Carbamazepine [2.5μg/ml in PBS]; Anastrozole – Tamoxifen [0.15μg/ml in PBS]), followed by 500μl diethylether (Fisher Scientific, UK): dichloromethane (Rathburn Chemicals Ltd, Scotland)(70:30). Tubes were then vortexed for 5 minutes, then centrifuged at 20,500 *x g* for 5 minutes in a benchtop centrifuge. The organic phase was carefully removed to a clean eppendorf and evaporated using the Eppendorf Concentrator Plus at 30°C for 30 minutes. The extracted drug was reconstituted in HPLC grade water:methanol (Letrozole – 60:40; Anastrozole - 50:50; Fisher Scientific, UK) and stored at -20°C before use.

2.3.3.2 HPLC Determination of Letrozole Concentration

Letrozole and carbamazepine (internal standard) were separated using an Agilent 1100 HPLC using a Hypersil ODS (5μm, 4.6 x 50mm; Agilent, UK) column maintained at 25**°C**. Initial chromatographic conditions were methanol:water (60:40)(methanol-Rathburn Chemicals Ltd, UK) at a flow rate of 1.0 ml/min. Mobile phase varied with time according to the protocol in **Table 2.3.3**. Letrozole and carbamazepine were determined by fluorescence ($\lambda_{Excitation}$ = 230nm, $\lambda_{Emmission}$ = 295nm) and UV (λ = 234nm) detection. Blood concentrations of letrozole were determined using the standard curve described in *section 2.3.3.1*.

Time /mins	Water (%)	Methanol (%)
0	40	60
4.90	40	60
4.91	5	80
6.00	5	80
6.01	40	60

Table 2.3.3: Elution profile for letrozole/carbamazepine chromatography

2.3.3.3 UPLC-MS-MS Determination of Anastrozole Concentration

UPLC-MS/MS analysis was performed using a Waters Acquity UPLC and Micromass Quattro Premier mass spectrometer (Micromass, Manchester, United Kingdom). Multiple reaction monitoring (MRM) data were acquired. The capillary voltage was set at 2kV, the desolvation temperature was at 450°C and source temperature at 120°C. Cone gas flow was 5 l/hr and desolvation gas flow was 800 l/hr. The cone voltage and collision energy were optimised for each compound, and are given in **Table 2.3.4**. A dwell time of 0.05 seconds between MRM transitions was used.

Substrate	MRM Transitions	Cone Voltage V	Collision Energy kV	Ion Mode
Anastrozole	286.2>217.2	25	15	ES +ve
Tamoxifen (IS)	372.2>71.9	40	26	ES +ve

Table 2.3.4: Modes of detection, machine parameters and chromatography used with UPLC-MS/MS

Samples and standards were diluted 1:10 prior to analysis in methanol:water (60:40). Anastrozole and internal standard (tamoxifen) were separated on an Acquity UPLC BEH C18 (1.7 µm, 2.1 x 50 mm) column, with an injection volume of 5µl and using a mobile phase of methanol:water (60:40 v/v). Elution was performed at a temperature of 45°C using the elution protocol given in **Table 2.3.5**, with eluent A being water + 0.1% formic acid (v/v) and eluent B being methanol + 0.1% formic acid (v/v). Transitions were made using a linear gradient. Blood concentrations of anastrozole were determined using the standard curve described in *section 2.3.3.1*.

Time min	Flow ml/min	Eluent A %	Eluent B %
Initial	0.6	40	60
0.30	0.6	10	90
0.65	0.6	10	90
0.70	0.6	40	60
1.00	0.6	40	60

Table 2.3.5: Elution profile for anastrozole/tamoxifen chromatography

2.3.3.4 Pharmacokinetic Parameter Analysis

Area under the curve (AUC), terminal half-life ($t_{½}$), maximum plasma concentration (C_{max}) and clearance were calculated in WinNonLin, version 3.1 (Pharsight) using a simple non-compartmental model.

2.3.4 CYP2B6-lacZ Reporter Analysis

Cryofixed livers generated in *section 2.2.3.5* were sectioned using a Bright Cryostat OFT5000 refrigerated microtome (15µm thickness), attached to

polysine microscope slides (VWR international, UK) and stored at -70°C prior to staining. On the day of staining, the sections were defrosted and air dried for 10 minutes, before being rehydrated in 2mM $MgCl_2$/PBS for 5 minutes. Sections were then covered in X-Gal (25mg/ml)/dimethylformamide solution and incubated at 37°C overnight. The following morning the X-Gal/dimethylformamide solution was discarded and the slides were washed for 5 minutes in 2mM MgCl2/PBS. They were then counter-stained with nuclear fast red dye (Vector Laboratories Ltd, UK) for 5 minutes and treated as follows: washed in distilled water, 4 minutes → washed in 70% ethanol, 4.5 minutes → washed in 95% ethanol, 1 minute → incubated in Histoclear II (National Diagnostics U.S.A., Georgia, USA), 3 minutes. The sections were then air dried for 5 minutes before the cover slip was attached using DPX mountant for histology. They were left at room temperature to cure for 5 days prior to microscopy.

Sections were visualized using a Zeiss Axio Scope.A1 microscope (Carl Zeiss Ltd, UK) at 10x, 40x and 100x magnification. Images were collected using Zeiss Axiovision Release 4.8.2 software (Carl Zeiss Ltd, UK). Densitometry analysis of LacZ staining was performed using the Axiovision Automeasure module (Carl Zeiss Ltd, UK).

2.3.5 Blood Biochemistry Analysis

Plasma supernatant generated in *section 2.2.3.5* was sent to The Clinical Pathology Service Laboratory, MRC Harwell (UK) for analysis of a panel of blood biochemistry markers, notably Creatinine, alkaline phosphatase (ALP), alanine transaminase (ALT), low density lipoprotein (LDL), glucose, total bilirubin and lactate dehydrogenase (LDH). Results were analysed for changes with respect to saline control.

CHAPTER 3

INITIAL SCREENING FOR PXR AND CAR INTERACTORS

3.1 INTRODUCTION

As discussed in *section 1.3*, PXR and CAR both have numerous physiological roles. Perhaps the most important is acting as co-ordinate regulators of drug metabolism, but others include the regulation of energy, cholesterol & bile acid metabolism, as well as inflammation. When considering these roles individually, it is clear that any drug that modulates the expression or activity of these receptors could cause potentially harmful adverse effects. When considering each receptor as part of an intricate network controlling multiple key physiological processes, the potential for, and severity of, adverse effects caused by PXR or CAR interaction must increase because of the range of disruptions that can occur. As a result, the risk of DDIs is also increased by PXR and CAR modulation. Identifying substances that modulate nuclear receptor function is therefore of key importance to drug development, and requires a variety of screening methodologies.

3.1.1 A QUESTION OF METHODOLOGY

Assessing the activation of nuclear receptors, in particular the modulation of PXR activity, in response to drug treatment is an important stage in early drug design. Several methodological approaches are now available to study nuclear receptor activation, including cell-based assays and transgenic animal studies. It is the high throughput *in vitro* assays that are used most widely in early drug design, with the many variants providing quick, quantitative indications about receptor activation.

3.1.1.1 TRANSACTIVATION ASSAYS

Transactivation (or reporter gene) assays are perhaps the most popular assay used when investigating drug interaction with nuclear receptors, and are responsible for identifying most of the currently available data regarding PXR and CAR activation **(Table 1.2.2)**. Indeed, transactivation assays examining PXR activation are routinely used by pharmaceutical companies to assess a novel compound's suitability for clinical development (Lin, 2006; Sinz et al.,

2008). These high-throughput assays are run in immortalized cell lines, such as the HepG2 hepatocellular carcinoma cell line, meaning that there is less variability in cell populations than is found in primary hepatocytes, another key model for the assessment of metabolic profiles. They also rely on a direct reporter to give data regarding activation potential, meaning that the effect can be quantified.

There are two main models employed when constructing transactivation assays **(Figure 3.1.1)**. Using the first method, the cells are transfected with the full-length nuclear receptor and a suitable reporter plasmid. The reporter plasmid commonly consists of a portion of target gene promoter (commonly *CYP3A4* in the case of hPXR or *CYP2B6* for hCAR) containing the DNA binding domain (DBD) of the nuclear receptor in question, linked to a measurable reporter gene, such as luciferase (Moore and Kliewer, 2000; Honkakoski et al., 2001; Luo et al., 2002; Lemaire et al., 2004; Yueh et al., 2005; Sinz et al., 2006; Harmsen et al., 2007; Cui et al., 2008; Yasuda et al., 2008; Harmsen et al., 2009; Shukla et al., 2011). This design provides good results regarding nuclear receptor activation potential caused by direct binding to the LBD. However, models employing this methodology compete with any innate nuclear receptor expressed by the cell, and although this is usually very low in immortalized cell lines, it could still interfere with reporter expression. The second method employs a chimeric plasmid containing the LBD of the nuclear receptor linked to the DBD of another protein, commonly the yeast transcription factor, GAL4. The chimeric plasmid is co-transfected with a reporter plasmid containing one or more copies of the DBD contained in the chimera, linked to, and driving the expression of, a measurable reporter, such as luciferase (Moore and Kliewer, 2000; Vignati et al., 2004; Mu et al., 2006; Shukla et al., 2011). The advantage of this design is that it removes the background noise caused by activating the innate nuclear receptor since the reporter plasmid can only be activated by the chimera (Moore and Kliewer, 2000).

Although useful to provide initial activation data for individual receptors, both types of assay suffer from significant disadvantages. A major shortcoming is the

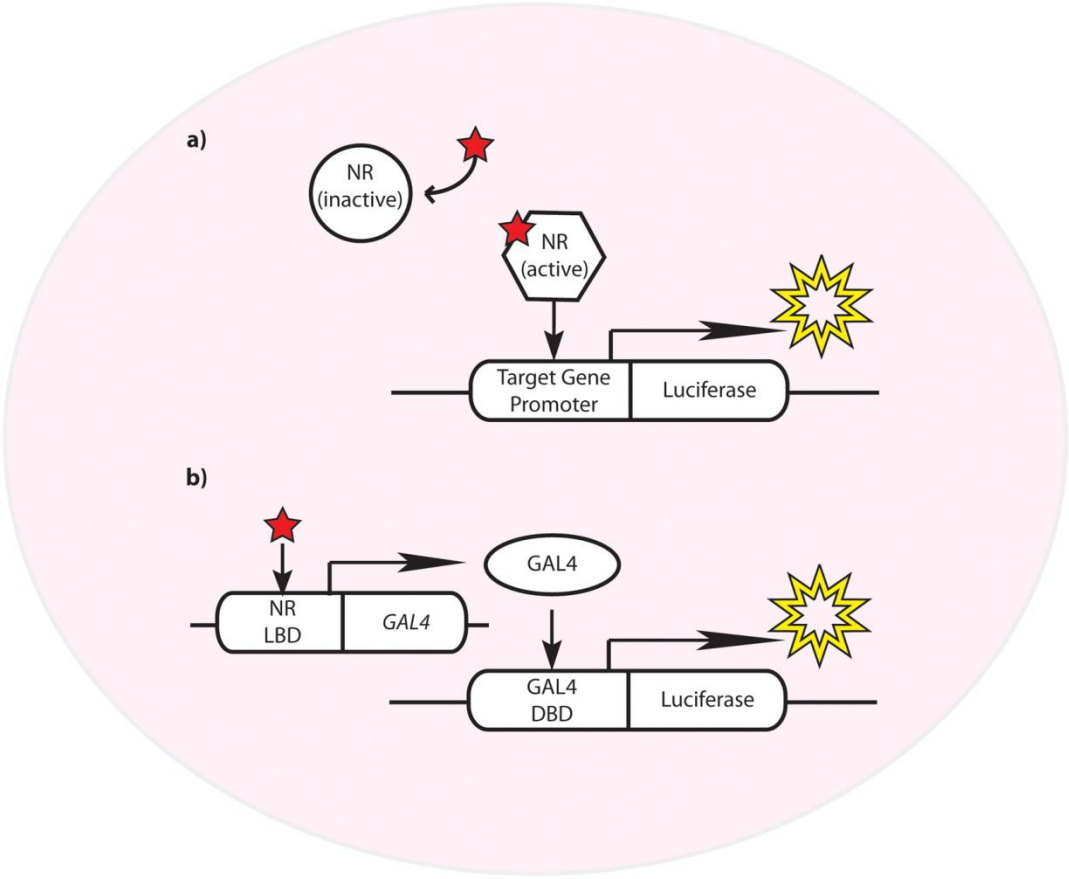


Figure 3.1.1: Two common methodologies for nuclear receptor transactivation assays

a) Full length nuclear receptor is transfected together with a reporter plasmid consisting of a fragment of target gene promoter containing nuclear receptor (NR) recognition site upstream of the luciferase reporter gene. Ligand binding induces binding to the NR recognition site and upregulates luciferase expression. b) A chimeric construct containing the NR ligand binding domain (LBD) linked to the GAL4 gene is transfected together with a reporter plasmid holding multiple repeats of the GAL4 DNA binding domain (DBD) upstream of the luciferase reporter. Ligand binding to the NR LBD induces GAL4 expression, and subsequently luciferase expression.

inability of either assay to model nuclear translocation, one of the key stages in the activation of target genes by PXR and CAR. The weakness of the first model lies in the overexpression of the full-length nuclear receptor. It is well documented that PXR and CAR have a propensity to automatically translocate to the nucleus, regardless of activation state, when overexpressed (Pascussi et

al., 2000; Zelko et al., 2001; Saradhi et al., 2005). This could potentially yield false positive results, positively identifying compounds that bind to the LBD when they don't activate gene transcription *in vivo* because they do not induce nuclear translocation. The second assay design uses only a fragment of the nuclear receptor, again creating an abnormal system in which LBD binding is segregated from nuclear translocation. This means that neither can model the full activation protocol of PXR and CAR. These assays are also unable to examine the role of nuclear receptor crosstalk, ignoring the complex network of transcription factors required for the correct regulation of target genes. This issue is exacerbated by the use of immortalized cell lines, in which expression levels of many transcription factors controlling drug metabolism are extremely low, removing any possibility of crosstalk between transcription factors. Experimental variation is significant in most assays of this type as a result of using transient transfection methods, with their associated differences in transfection efficiency (Plant et al., 2000). However, some groups have overcome this variability by creating stably transfected cell lines (Honkakoski et al., 2001; Raucy et al., 2002; Lemaire et al., 2004). Finally, cellular isolation from systemic cues, such as hormonal signalling and energy state, removes a key level of transcriptional control with respect to PXR and CAR activation, with several endogenous compounds being identified as modulating their activity **(Table 1.2.2)**.

3.1.1.2 PRIMARY HUMAN HEPATOCYTE MODELS

Primary human hepatocytes (PHH) are the gold standard for *in vitro* pre-clinical drug tests, displaying full metabolic functionality and having the closest relationship to clinical data. They are therefore considered to be a good model for pre-clinical assessment of drug interactions by the FDA (Guguen-Guillouzo and Guillouzo, 2010), giving data including enzyme expression and activity, gene expression and pharmacokinetics. However, when considering a model for nuclear receptor activation studies, there are significant disadvantages to the basic PHH methodology. General issues associated with culturing PHH include being costly, difficult to maintain, unpredictable to source and having a limited shelf life before their metabolic profile starts to degrade (Brandon et al.,

2003; Harmsen et al., 2007; Guguen-Guillouzo and Guillouzo, 2010). Inter-individual variation is also a problem, since it is extremely unlikely all experiments can be sourced from the same individual, and therefore hepatocytes from other donors are likely to be required (Brandon et al., 2003; Guguen-Guillouzo and Guillouzo, 2010; Kamiguchi et al., 2010). Although the ability to select hepatocytes displaying a similar metabolic profile and the use of pooled hepatocyte populations does ameliorate this effect to a certain extent, it cannot entirely remove it (Brandon et al., 2003; Kamiguchi et al., 2010).

An issue specific to nuclear receptor activation analysis is the lack of a convenient direct reporter innate to the PHH model. Examining cytochrome P450 expression patterns can give a strong indication of which receptors are likely to have been activated, but they cannot quantify activation or specifically dissect which receptors are involved because of the complex crosstalk required for transcriptional regulation. Another shortcoming of the PHH model is that the expression of hCAR is rapidly down-regulated following harvest, with levels being commensurate with those in human liver samples for up to four days before being very significantly reduced (Pascussi et al., 2000; Washio et al., 2011). The basic primary human hepatocyte model is therefore not necessarily the first choice when screening drugs for nuclear receptor interaction, and must be combined with other techniques, such as transactivation assays or transgenic *in vivo* models to give meaningful data. However, technological advances have resulted in PHH models with greater utility for the analysis of nuclear receptor activation. The use of siRNA technology to produce knockdown models for the nuclear receptors allows the complex transcriptional control circuitry to be dissected, and consequently gives greater confidence in data obtained from the basic PHH model (Sane et al., 2008a). Another model which has recently emerged involves the adenoviral transduction of a functional enhanced yellow fluorescent protein linked hCAR (EYFP-hCAR) protein into PHH (Li et al., 2009a; Tolson et al., 2009). This protein localises in the cytoplasm and subsequently translocates to the nucleus following direct activation. It is therefore possible to examine the nuclear translocation of hCAR

following drug treatment in a real-time manner using a convenient reporter, and negates some of the limitations of the native PHH model.

3.1.1.3 IN VIVO MODELS

Whilst *in vitro* techniques are powerful tools that produce fast, high-throughput screening data regarding the nuclear receptor activation potential of a chemical, they all have limitations when trying to analyse systems level phenomena. The inability of the majority of *in vitro* assays to model nuclear translocation, one of the key and characteristic stages in nuclear receptor activation, is a significant weakness. Another key problem lies in the isolation of cultured cell populations from systems level modulators, including hormones and dietary factors, which are known to have an effect on the activation status of nuclear receptors. Although culture conditions can be modified to reflect specific changes in hormone levels, etc., they cannot reflect the full complexity of external cellular modulators. If a more complete understanding of nuclear receptor behaviour in a system is required, more complex non-human *in vivo* models must be used.

As with all methodologies, the *in vivo* models are not infallible, and suffer from their own limitations. There are ethical and legal issues associated with their use, covered by The Animals (Scientific Procedures) Act 1986, which are avoided by using *in vitro* methods. Experiments run *in vivo* are much slower than the high-throughput *in vitro* methods, although they are able to yield significantly more data from a single experiment with efficient method design. Because of the cost of maintenance, these models are costly to run. However, the most important drawback of using non-human *in vivo* models is that of species-specificity. This phenomenon will be further discussed in *Chapter 4.1.2.1*, but in brief describes the differences in activation potential between human and non-human receptors, resulting in differing metabolic profiles. It is a key consideration when using *in vivo* models, and requires careful experimental design to obtain meaningful data.

Although there are several experimental considerations associated with the use of these models, with correct model selection inferences can be made that are of material advantage when progressing studies into clinical models. These are

the only pre-clinical models that are able to fully consider the role of systems level modulators in receptor function, and as such provide benefits that can't be achieved using a cell based methodology. Although wild-type animals are commonly used for initial toxicity and pharmacokinetic screens, there are other, more sophisticated *in vivo* methodologies available to analyse specific aspects of drug metabolism. One example is the use of xenograft models, in which a human cell line is implanted into an immunocompromised animal (commonly mouse), to analyse drug-induced tumour necrosis. These models provide greater clinical relevance because of the use of human cell lines, and give initial data regarding drug efficacy. However, these models still rely on innate mouse physiology and therefore any information regarding drug metabolism must be treated with care. The advent of transgenic models has meant that *in vivo* experiments can now be designed to specifically dissect drug metabolism and its control. They are of particular use for analysing receptor activation and inter-species variation, with models available in which various constituents of the drug metabolism network are removed or humanized, enabling drug-induced transcriptional control to be assessed. These will be discussed in more detail in *Chapter 4*.

3.1.1.4 Aims and Objectives

In order to study the role of PXR and CAR in drug-drug interactions, we selected an *in vivo* methodology using a panel of novel PXR and CAR transgenic mice (Scheer et al., 2008; Ross et al., 2010; Scheer et al., 2010). These will allow us to dissect the control of drug metabolism in response to drug treatment with respect to the two nuclear receptors, and give us systems level information regarding gene expression, Cytochrome P450 enzyme activity and changes in drug pharmacokinetics. Prior to testing in the transgenic models, preliminary investigations were performed in C57BL/6J wild-type mice, the background upon which the transgenic mice are based. Firstly, in order to select an experimental drug, several currently available anti-cancer drugs were tested for cytochrome P450 modulation **(Table 3.1.1)**, particularly induction or repression of Cyp3a11 and Cyp2b10 which are prototypically modulated by PXR and CAR respectively. All of these drugs were selected as potential PXR

Drug	Mechanism	Indication	Clinical Dose	Cytochrome P450s metabolised by	
Anastrozole	Aromatase Inhibitor (selective, non-steroidal)	*ER-positive breast cancer (post-menopausal women)* ➢ Early adjuvant therapy (sole) ➢ Early adjuvant therapy (post Tamoxifen) ➢ Advanced adjuvant therapy	1mg p.o. daily for 5 years	CYP3A4 CYP3A5 (minor) *Kamdem *et.al* (2010)	
Cyclophosphamide	Alkylating agent	*Wide range of malignancies, including:* ➢ Lymphomas/Leukaemias ➢ Multiple myeloma ➢ Ovarian Adenocarcinoma ➢ Breast carcinoma	3-50mg/kg I.V. OR 1-5mg/kg/day p.o.	CYP2A6 CYP2B6 CYP2C8 CYP2C9	CYP2C19 CYP3A4 CYP3A5
				http://www.pharmgkb.org/pathway/PA2034#PGG	
Gefitinib	Tyrosine kinase inhibitor	Locally advanced/metastatic non-small cell lung carcinoma with epidermal growth factor receptor-tyrosine kinase mutations	250 mg p.o. daily	CYP3A4 CYP3A5	CYP1A1 CYP2D6
				*Duckett and Cameron (2010)	
Letrozole	Aromatase Inhibitor (selective, non-steroidal)	*ER-positive breast cancer (post-menopausal women)* ➢ Early adjuvant therapy (sole) ➢ Early adjuvant therapy (post-Tamoxifen) ➢ Locally advanced/ metastatic first-line therapy ➢ Advanced with disease progression post-Tamoxifen	2.5mg p.o. daily for 5 years	CYP2A6 CYP3A4 *Murai *et.al* (2009)	

Table 3.1.1: Anti-cancer drugs used in initial drug screen

inducers because of their reported induction of CYP3A4 (Scripture et al., 2005; Purnapatre et al., 2008). Having chosen our experimental drugs, we thenperformed experiments to optimise the dose of drug required to give an acceptable level of gene expression prior to testing in the transgenic models.

3.2 METHODS: *IN VIVO*

3.2.1 ANTI-CANCER DRUG PANEL TREATMENT

Mice were dosed with a panel of anti-cancer drugs reported to be associated with increased expression or activity of the Cyp3a or Cyp2b enzymes, which are known to be prototypically induced by PXR and CAR respectively. C57 BL/6J wild type mice (n=3 per group; male and female) were dosed *p.o.* on a once daily basis for 3 days with anastrozole (0.3 mg/kg), cyclophosphamide (50 mg/kg), gefitinib (100 mg/kg) letrozole (100 mg/kg), or an equivalent volume of 0.9% saline vehicle control. Mice were euthanized using rising CO_2 24 hours following final dose.

Following euthanasia, blood was harvested by cardiac puncture into heparinized tubes which were subsequently centrifuged at 16,000 *x g* for 10 minutes. Plasma supernatant was removed to a clean eppendorf which was snap frozen in liquid N_2 and stored at -70˚C prior to analysis. Liver was weighed, then one small piece of liver was harvested into an eppendorf tube, with the remainder being bisected and transferred into bijou tubes. All liver samples were snap frozen in liquid N_2 and stored at -70˚C prior to analysis.

3.2.2 AROMATASE INHIBITOR DOSE RESPONSE ANALYSIS AND PHARMACOKINETIC STUDY

To allow dose selection for future studies by comparison with samples generated in the anti-cancer panel study, C57BL/6J wild type mice (n=3 per group; male and female) were dosed *p.o.* on a once daily basis for either 1 or 3

days with anastrozole (10 mg/kg or 20 mg/kg), letrozole (25 mg/kg or 50 mg/kg), or an equivalent volume of 0.9% saline vehicle control.

In the 24 hours following the final dose, the drug-treated animals were periodically restrained to enable serial blood sampling from the tail vein. One incision only was made into the tail vein at the start of the procedure, with the wound being periodically reopened throughout the day by warming using water, taking care to stop the bleeding immediately following sampling using manual pressure. Blood (10μl) was sampled at 10, 30, 60, 180, 360, 480, 720 and 1440 minutes after dosage using a Gilson pipette into a clean eppendorf tube containing an equivalent volume of heparin (15 IU/ml) and kept on ice over the day, prior to long term storage at -70˚C. For letrozole, 5μl blood was collected because of the higher sensitivity of the downstream letrozole assay. Mice were euthanized using rising CO_2 following the final 24 hour timepoint.

Following euthanasia, blood was harvested by cardiac puncture into heparinized tubes which were subsequently centrifuged at 16,000 *x g* for 10 minutes. Plasma supernatant was removed to a clean eppendorf which was snap frozen in liquid N_2 and stored at -70˚C prior to analysis. Liver was weighed, then one small piece of liver was harvested into an eppendorf tube, with the remainder being bisected and transferred into bijou tubes. All liver samples were snap frozen in liquid N_2 and stored at -70˚C prior to analysis.

3.2.3 Sample Processing and analysis

All liver samples generated by the *in vivo* experiments described below were processed and analysed as described in *Sections 2.3.1* and *2.3.2.*

3.3 RESULTS

3.3.1 SCREENING THE ANTI-CANCER DRUG PANEL

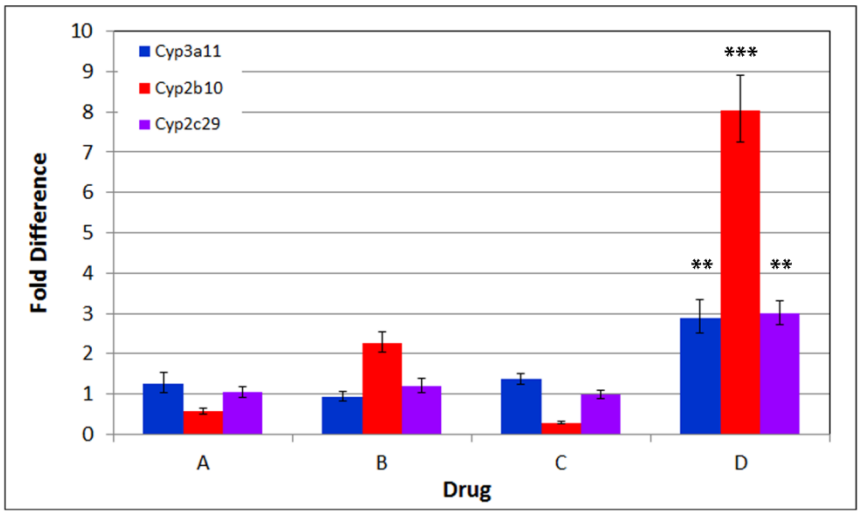
3.3.1.1 ANALYSING GENE EXPRESSION FOLLOWING DRUG TREATMENT

As a first step to probing the potential for PXR and CAR interaction, whole liver mRNA from vehicle control and drug treated mice was analysed by TaqMan® real-time PCR for the expression of *Cyp3a11, Cyp2b10* and *Cyp2c29*, all of which are targets of these nuclear receptors. At the doses tested, anastrozole and gefitinib did not significantly affect the expression of any of the genes in males or females **(Figure 3.3.1)**. The oxazaphosphorine cyclophosphamide had no effect on *Cyp3a11* or *Cyp2c29* expression in either sex. However, a trend towards higher *Cyp2b10* expression following cyclophosphamide treatment was seen in male mice, although it was not significant. This is in agreement with published data which indicates that cyclophosphamide auto-induces its own metabolism by CYP2B6 through interaction with CAR and PXR (Chang et al., 1997; Lindley et al., 2002; Harmsen et al., 2009; Wang et al., 2011). In both sexes, very significant increases in expression of *Cyp3a11* were seen following treatment with letrozole, and extremely significant induction of *Cyp2b10*. There was also induction of *Cyp2c29*, although this was only significant in the male population. This suggests that letrozole is a promising candidate to interact with both nuclear receptors, and particularly with CAR, the receptor which prototypically induces *Cyp2b10*.

3.3.1.2 LETROZOLE INDUCES NUMEROUS CYTOCHROME P450S

Although gene expression data suggests that induction of cytochrome P450s is occurring in response to drug treatment, it is of importance to know if increases in mRNA are associated with a corresponding induction in functional protein. Western blots were performed using pooled microsomal fractions isolated from whole liver samples harvested from treated mice. These were then probed with polyclonal antibodies raised against a variety of key cytochrome P450s together with cytochrome P450 oxidoreductase (POR).

a)



b)

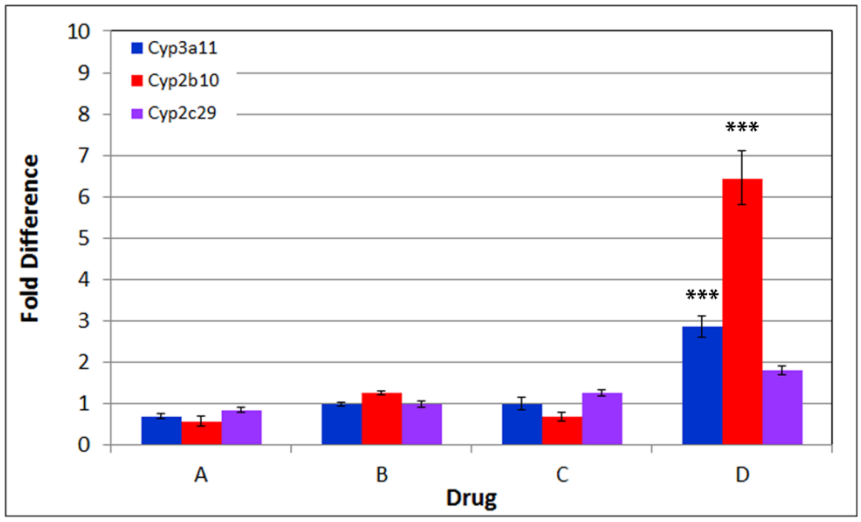
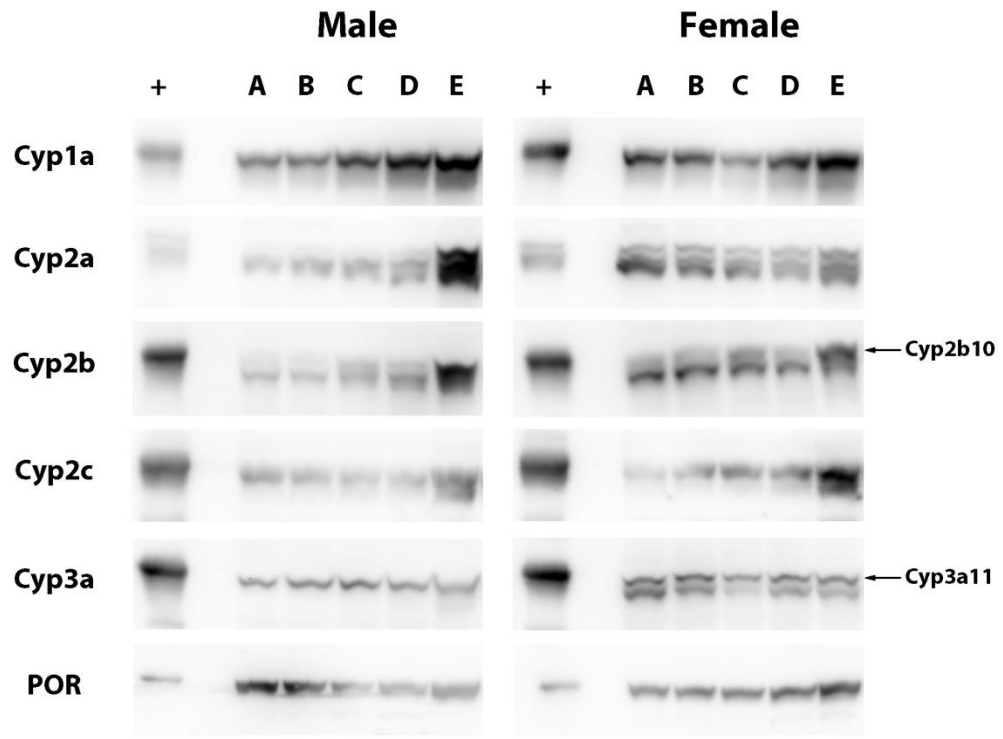


Figure 3.3.1: Induction of Cytochrome P450 mRNA in wild type C57BL/6J mice following 3 day treatment with an anti-cancer drug panel

*C57BL/6J mice (n=3 per group) were treated daily with either 0.9% Saline, 0.3 mg/kg Anastrozole (A), 50 mg/kg Cyclophosphamide (B), 100 mg/kg Gefitinib (C) or 100 mg/kg Letrozole (D) for 3 days. Cyp3a11, Cyp2b10 and Cyp2c29 mRNA expression relative to 0.9% Saline vehicle control was measured by TaqMan gene expression analysis in male (a) and female (b) liver RNA, and is expressed as fold difference ± range incorporating S.D. as recommended by Applied Biosystems comparative Ct analysis method. Statistics analysed gene expression following treatment vs vehicle control. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.*

a)



b)

Drug treatment	Fold Difference												
	Male						Female						
	Cyp1a	Cyp2a	Cyp2b	Cyp2c	Cyp3a	POR	Cyp1a	Cyp2a	Cyp2b	Cyp2c	Cyp3a (Upper)	Cyp3a (Lower)	POR
0.9% Saline (A)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Anastrozole (B)	1.1	1.4	1.0	0.8	1.4	0.9	0.9	0.8	1.1	1.6	1.0	0.8	1.1
Cyclophosphamide (C)	1.7	1.6	1.5	0.7	1.5	0.4	0.6	0.6	1.0	2.1	0.6	0.5	1.2
Gefitinib (D)	2.2	2.3	2.1	0.8	1.2	0.4	1.2	0.6	0.8	2.6	0.8	0.6	1.5
Letrozole (E)	2.5	8.1	4.2	1.9	1.3	0.6	1.5	0.9	1.2	6.1	0.8	0.6	2.0

Figure 3.3.2: Induction of Cytochrome P450s in wild type C57 BL/6J mice following 3 day treatment with an anti-cancer drug panel

C57BL/6J mice (n=3 per group) were treated daily with either 0.9% Saline (A), 0.3 mg/kg Anastrozole (B), 50 mg/kg Cyclophosphamide (C), 100 mg/kg Gefitinib (D) or 100 mg/kg Letrozole (E) for 3 days. a) Western blots of pooled liver microsomes (n = 3 per group; 20μg/well). + = positive control - Liver microsomes from male C57 BL/6J mouse treated with either phenobarbital (80 mg/kg/day for 3 days; 7.5μg/well; Cyp1a, 2a, 2b, and 2c) or dexamethasone (50 mg/kg/day for 3 days; 5μg/well; Cyp3a and POR). b) Densitometry analysis - Fold induction/repression following drug treatment relative to saline control. Numbers in red indicate >2 fold induction, numbers in green indicate >2 fold repression. Figures for Cyp1a, Cyp2a and Cyp2c represent densitometry for all bands identified on blot. Cyp2b and Cyp3a indicate densitometry for Cyp2b10 and Cyp3a11 isoforms only.

At a glance, it can be seen that none of the drugs significantly affect Cyp3a expression in mice, a surprising finding since CYP3A4 is involved in the metabolism of all four **(Figure 3.3.2)**. It is of particular interest in the case of letrozole, because it suggests that the increased level of mRNA identified by TaqMan® analysis does not translate to a corresponding induction in protein. It is also clear that anastrozole does not affect cytochrome P450 expression at the dose tested. With respect to the other drugs, a clear increase in Cyp1a, Cyp2a and Cyp2b expression is seen in male mice following treatment with cyclophosphamide and gefitinib, although only gefitinib achieves a >2 fold increase. However, the situation is more equivocal when considering expression in female mice, with the higher basal expression of cytochrome P450s in females negating equivalent inductions. Unlike in males, there is no induction of Cyp1a or Cyp2a in females. There is also a modest induction of Cyp2b10 (the mouse orthologue to CYP2B6, and the top band detected by the antibody) in females following treatment with cyclophosphamide, but not with gefitinib. Unfortunately immunoblot resolution is insufficient to allow quantitation of the Cyp2b10 isoform, and therefore this induction is masked when assessing densitometric data from the whole cluster. There are also gender discrepancies when analysing Cyp2c expression, with there being no expression following gefitinib or cyclophosphamide treatment in males, but significant inductions with the same treatment in females (2.6 and 2.1 fold respectively, relative to vehicle treated).

To this point letrozole induction has not been discussed. Looking at the Western blots, it is immediately evident that letrozole strongly induces the expression of Cyp1a, Cyp2a and Cyp2b enzymes in male mice, with 2.5-, 8.1- and 4.2-fold induction respectively being seen upon densitometric analysis. A Cyp2c induction of 1.9 fold is also seen in males; there is no induction of Cyp3a or POR. In females, modest inductions of Cyp1a, Cyp2a and Cyp2b can be seen, although none of these reach the >2 fold threshold. The most significant induction in females following letrozole treatment was in Cyp2c protein, where a 6.1-fold induction was measured. Interestingly, letrozole was also responsible for a female-specific 2-fold induction in POR, the enzyme required for all

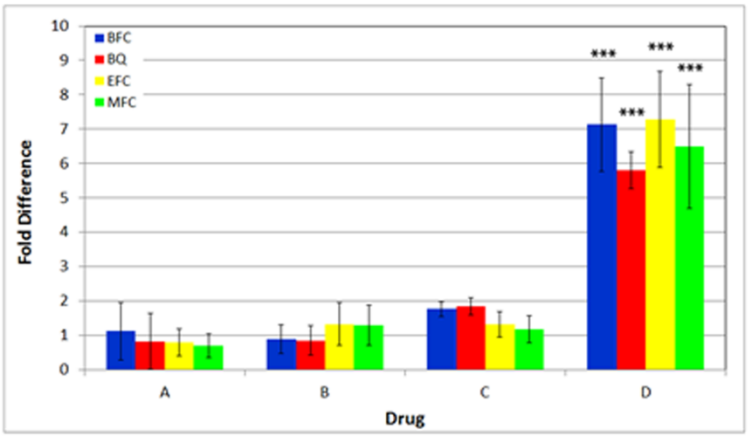
cytochrome P450 reactions. On the basis of these results, it is clear that letrozole provides the best protein induction of all drugs tested, and is a potential substrate for PXR and CAR. It is also evident that sexual dimorphism in cytochrome P450 expression and induction is an issue that must be considered in further experimental design.

3.3.1.3 Letrozole induces Cytochrome P450 activity

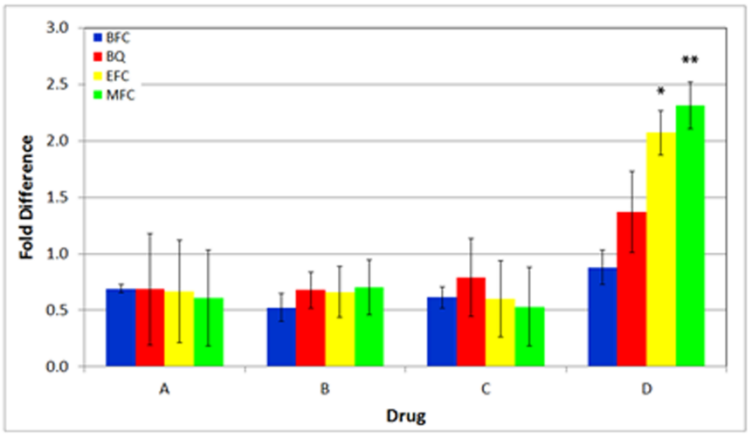
Western blot analysis showed that cytochrome P450s were being induced in response to drug treatment. However, the most crucial aspect of protein induction with respect to drug-drug interactions is that of enzyme activity. As part of this preliminary investigation, a panel of coumarin probes were used to measure isoform activity **(Table 2.3.2)**.

Data indicated that there was no significant induction in enzyme activity following anastrozole, cyclophosphamide or gefitinib treatment in males or females, although there was a non-significant increase of approximately 2-fold in BFC and BQ activity after gefitinib treatment in males **(Figure 3.3.3)**. This primarily indicates an increase in Cyp3a activity, although Cyp1a activity is also likely to be involved. An increase in Cyp1a activity coincides with the increased induction seen in the Western blots. Once again, letrozole induced the most profound changes in enzyme activity. In males, activity data from all four probes is significantly increased by between 6 and 7-fold, suggesting a broad spectrum induction of enzyme activity, including Cyp1a, Cyp2b, Cyp2c and Cyp3a. Again, there is sexual dimorphism in the response to letrozole. No induction was seen in females with BFC and BQ probes, indicating that Cyp3a and Cyp1a enzymes are not being induced, an observation which concurs with the Western blots. However, significant increases in EFC and MFC activity indicate that Cyp2b, Cyp2c and Cyp2e enzymes are being induced. Once again, the activity induction profiles suggest a role for CAR and PXR in the control of letrozole-induced metabolism.

a)



b)



Substrate (S)	Enzyme detected
7-benzyloxy-4-trifluoromethyl-coumarin (BFC)	CYP3A4 CYP1A2 CYP2C19 CYP1B1 CYP1A1
7-ethoxy-4-trifluoromethyl-coumarin (EFC)	CYP2B6 CYP2C19 CYP2E1
7-methoxy-4-trifluoromethyl-coumarin (MFC)	CYP2C9 CYP2B6 CYP2E1
7-benzyloxy-quinoline (BQ)	CYP3A4 CYP1A1/2

Figure 3.3.3: Cytochrome P450 activity in wild type C57 BL/6J mice following 3 day treatment with an anti-cancer drug panel

*C57 BL/6J mice (n=3 per group) were treated daily with either 0.9% Saline, 0.3 mg/kg Anastrozole (A), 50 mg/kg Cyclophosphamide (B), 100 mg/kg Gefitinib (C) or 100 mg/kg Letrozole (D) for 3 days. Cytochrome P450 activity in male (a) and female (b) liver microsomes was assessed using a panel of coumarin probes (human specificity given in table). Data is expressed as fold difference ± S.D. Statistics analysed average specific activity following treatment vs vehicle control. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.*

3.3.2 OPTIMIZING THE DOSE OF AROMATASE INHIBITORS

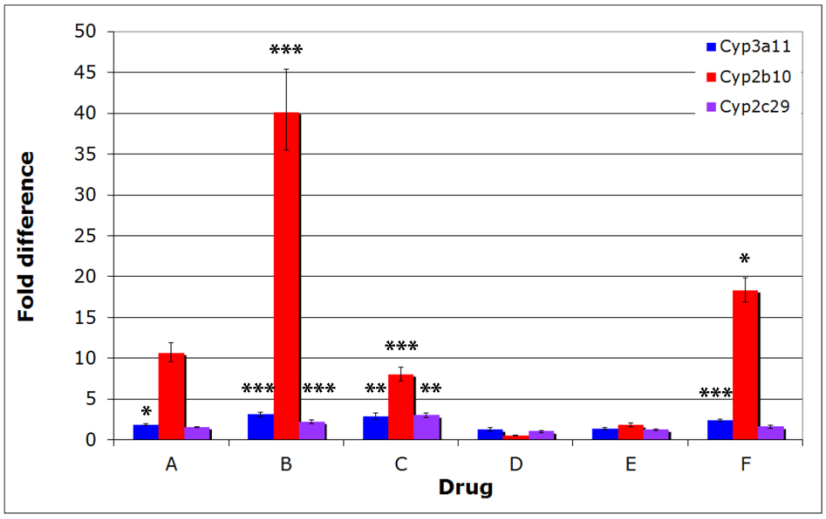
On the basis of gene expression, Western blot and enzyme activity data, it was decided that letrozole was a likely target of CAR and PXR. However, the dose used in the initial screen was very high in comparison to doses used clinically (100mg/kg vs 2.5 mg/day (approx. 0.038mg/kg based on an average 65kg woman)), and therefore experiments were performed to identify a lower dose which would give acceptable expression of cytochrome P450s. As a result of the strength of induction following letrozole treatment, it was also decided to re-analyse induction caused by the aromatase inhibitor anastrozole. In the initial study, the dose tested was very low (0.3mg/kg) because experience within the lab indicated that this dose gave good data in pharmacokinetic studies. However, the low dose may have contributed to the inability to identify any changes in cytochrome P450 expression, and therefore the dose was increased in the current cohort.

3.3.2.1 GENE EXPRESSION IS INDUCED FOLLOWING AROMATASE INHIBITOR TREATMENT

Following experiences in the initial study, two lower doses of letrozole were tested: 25mg/kg and 50mg/kg. Anastrozole doses were increased to 10mg/kg and 20mg/kg. Although these are significantly higher than the clinical dose, we decided that they were most appropriate for an initial "proof of process" study, and could be reduced at a later date. TaqMan® data for the new doses was analysed alongside the data generated from the original test cohort, and is shown in **Figure 3.3.4**.

As before, *Cyp2b10* is strongly upregulated following letrozole treatment in both males and females, although there is no dose-dependent induction. There is sexual dimorphism in the magnitude of induction relative to vehicle control, with induction in males ranging between 10 and 50-fold, whereas induction in females is more modest, at between 4 and 6.5-fold. This is as a result of the higher basal expression of *Cyp2b10* in the females. However, the inductions are

a)



b)

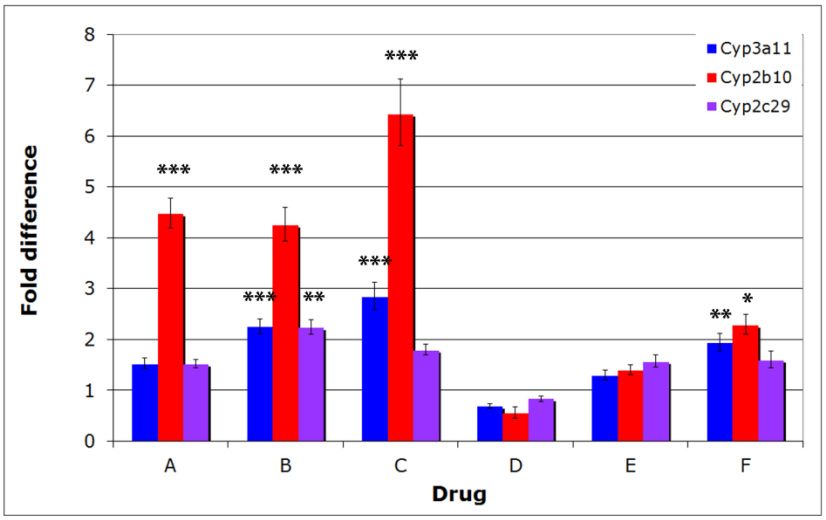


Figure 3.3.4: Induction of Cytochrome P450 mRNA in wild type C57 BL/6J mice following 3 day treatment with various doses of Letrozole and Anastrozole

*C57 BL/6J mice (n=3 per group) were treated p.o for 3 days with either 0.9% Saline, Letrozole (25 (A), 50(B) or 100 (C) mg/kg/day) or Anastrozole (0.3 (D), 10 (E) or 20 (F) mg/kg/day). Cyp3a11, Cyp2b10, and Cyp2c29 mRNA expression relative to 0.9% Saline vehicle control was measured by TaqMan gene expression analysis in male (a) and female (b) liver RNA, and is expressed as fold difference ± range incorporating S.D. as recommended by Applied Biosystems comparative Ct analysis method. Statistics analysed gene expression following treatment vs vehicle control. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.*

considered to be highly significant ($p \leq 0.001$) in both males and females, with the exception of the 25mg/kg dose in males.

Cyp3a11 is also significantly induced in all treatment groups except the 25mg/kg dose in females. Unlike *Cyp2b10* the effect appears to be dose-dependent in the female cohort, varying from 1.5-fold in the 25mg/kg group to 2.8-fold in the 100mg/kg cohort. In the males, all doses significantly upregulated *Cyp3a11* expression, with an increase from 1.9-fold in the 25mg/kg group to 3.1-fold in the 50mg/kg group being seen. Induction then plateaus between 50-100mg/kg, with the latter being induced 2.9-fold. This suggests that maximal induction of *Cyp3a11* is achieved between 25-50mg/kg treatment indices in males, but that further induction is possible in females.

Although induction of *Cyp2c29* is seen in the 25mg/kg dosing group in both sexes (1.6-fold in males, 1.5-fold in females), it is not considered significant. However, dose-dependent upregulation is observed in males, with induction increasing to 2.2-fold after 50mg/kg treatment, and then to 3-fold following 100mg/kg. In females, no dose-dependency is seen, with a significant induction to 2.2-fold being observed after 50mg/kg treatment, followed by a non-significant induction of 1.8 fold after 100mg/kg treatment. In contrast to findings with *Cyp3a11*, it appears that maximal *Cyp2c29* induction is achieved between 25-50mg/kg treatment indices in females, but that further induction is still possible in males. It also suggests that the induction mechanism for the *Cyp3a11* and *Cyp2c29* genes is sexually dimorphic, a finding that has been previously reported in the literature (Down et al., 2007; Hernandez et al., 2009b).

When analysing the data following anastrozole treatment, it can be seen that there is little induction at doses lower than 20mg/kg, with only *Cyp2b10 (male)* and *Cyp2c29 (female)* approaching significance (1.8- and 1.6-fold respectively). Using a 20mg/kg dosing regime, there is very significant induction of *Cyp3a11* in both males and females (2.4- and 1.9-fold respectively). This correlates well with the identification of CYP3A4 as the key cytochrome P450 involved in the metabolism of anastrozole (Kamdem et al., 2010), and suggests that anastrozole

may auto-induce its own metabolism. A significant upregulation of *Cyp2b10* is observed in both males and females, although the magnitude of induction appears lower in females. Again, this may be as a result of the higher basal expression of *Cyp2b10* in females, with actual expression level being approximately twice that of the males (data not shown). There is no significant induction of *Cyp2c29* in either sex following treatment with 20mg/kg anastrozole.

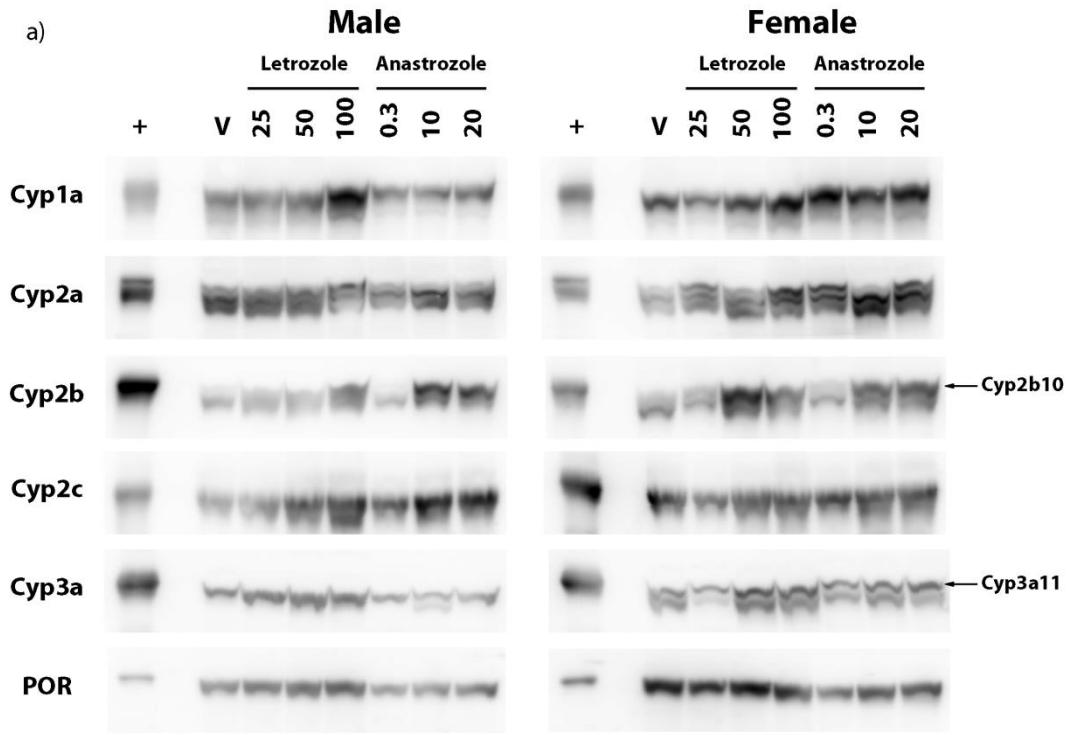
3.3.2.2 Both aromatase inhibitors induce Cytochrome P450 protein expression

To assess protein expression, a series of Western blots were performed on pooled microsomal extracts isolated from whole liver samples harvested from treated mice. These were probed using a panel of polyclonal cytochrome P450 antibodies together with a polyclonal antibody for POR. Only 100mg/kg letrozole induced Cyp1a in males and females, with the lower doses having little effect **(Figure 3.3.5)**. However, sexually dimorphic expression of Cyp1a was observed following anastrozole treatment, with no induction being seen in males and slight induction being seen in females at all doses.

The expression of Cyp2b is also influenced by letrozole treatment, with induction being seen following 100mg/kg treatment in males, but after 50mg/kg in females. This indicates that Cyp2b expression is more sensitive to letrozole-induced upregulation in females than in males. On the other hand, anastrozole induces Cyp2b expression in both the 10 and 20mg/kg treatment groups in both sexes. However, the magnitude of induction is greater in males than in females (3- and 2.7-fold vs 1.7- and 1.9-fold respectively) as a result of the higher basal expression rate in females.

Sexual dimorphism was seen in the expression of Cyp2a, with no induction in males with either drug, but induction being seen with both drugs at all doses in females (2.1- and 2.6-fold for letrozole, 2.7-3.1-fold for anastrozole). The 25mg/kg letrozole treatment group did not reach the 2-fold threshold, although it was induced by 1.8-fold. It is also noteworthy that anastrozole at all doses induced Cyp2a more strongly than letrozole, even at the highest dose. In

a)



b)

Drug treatment	Fold Difference												
	Male						Female						
	Cyp1a	Cyp2a	Cyp2b	Cyp2c	Cyp3a	POR	Cyp1a	Cyp2a	Cyp2b	Cyp2c	Cyp3a (Upper)	Cyp3a (Lower)	POR
0.9% Saline (V)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Letrozole (25 mg/kg)	1.2	1.0	1.3	1.4	1.5	1.3	0.8	1.8	1.0	0.6	0.8	0.6	0.9
Letrozole (50 mg/kg)	1.4	1.0	1.2	2.3	1.7	1.4	1.1	2.1	2.4	1.0	1.5	1.8	1.1
Letrozole (100 mg/kg)	2.2	0.8	2.2	3.0	1.5	1.6	1.5	2.6	1.6	1.0	1.7	1.4	0.8
Anastrozole (0.3 mg/kg)	0.9	0.7	0.9	2.0	0.8	0.8	1.5	2.7	0.9	0.9	1.0	1.1	0.5
Anastrozole (10 mg/kg)	0.8	0.9	3.0	2.9	0.7	0.9	1.4	3.1	1.7	1.1	1.2	1.4	0.7
Anastrozole (20 mg/kg)	1.0	0.8	2.7	2.8	0.9	1.2	1.5	3.1	1.9	1.1	1.2	1.0	0.7

Figure 3.3.5: Dose dependent induction of Cytochrome P450s in wild type C57 BL/6J mice following 3 day treatment with Letrozole or Anastrozole

C57 BL/6J mice (n=3 per group) were treated p.o for 3 days with either 0.9% Saline (V), Letrozole (25, 50 or 100 mg/kg/day) or Anastrozole (0.3, 10 or 20 mg/kg/day). a) Western blots of pooled liver microsomes (n = 3 per group; 20μg/well). + = positive control - Liver microsomes from male C57 BL/6J mouse treated with either phenobarbital (80 mg/kg/day for 3 days; 7.5μg/well; Cyp1a, 2a, 2b, and 2c) or dexamethasone (50 mg/kg/day for 3 days; 5μg/well; Cyp3a and POR). b) Densitometry analysis - Fold induction/repression following drug treatment relative to saline control. Numbers in red indicate > 2 fold induction. Densitometry for Cyp1a, Cyp2a and Cyp2c represents expression of full gene cluster. Densitometry for Cyp2b and Cyp3a represents expression of Cyp2b10 and Cyp3a11 isoforms only.

contrast, Cyp2c is induced following the two highest doses of letrozole and anastrozole in males (2.3- and 3-fold vs 2.9- and 2.8-fold respectively), but is essentially unchanged in females.

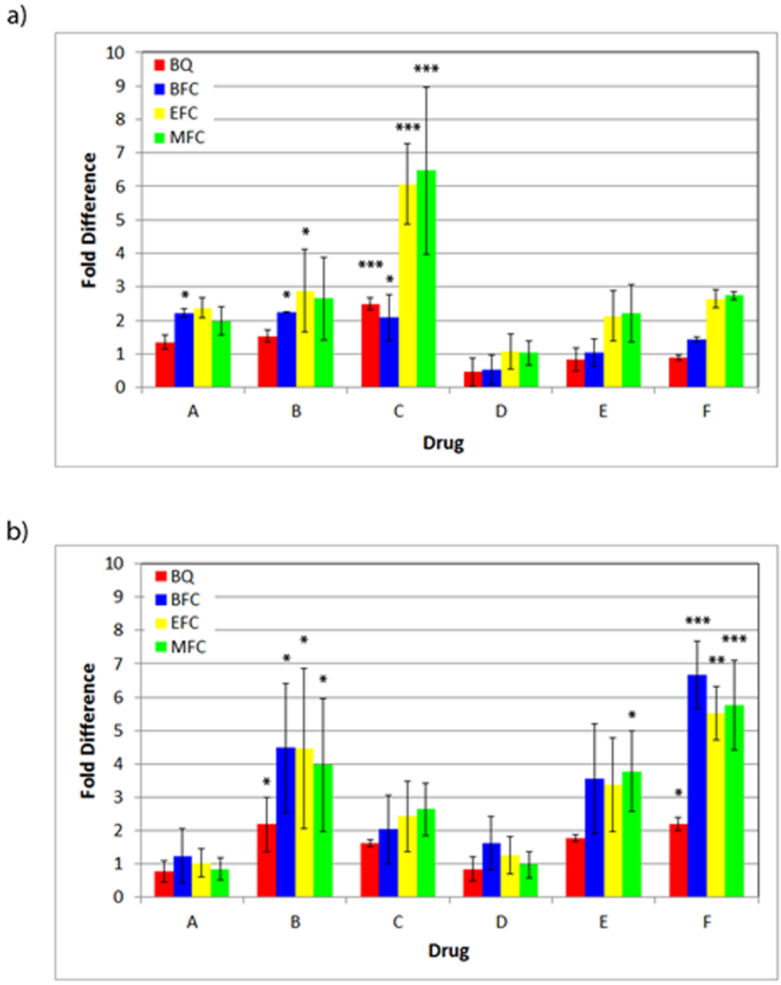
With respect to Cyp3a, a key enzyme in the metabolism of both drugs, no induction is seen at any of the doses tested in both sexes. However, a modest induction of less than the two fold threshold (1.5-1.7 fold) is seen following letrozole treatment in males and females. The exception to this is observed at the lowest drug concentration in females, where no induction is recorded. These findings correlate well with the data obtained by TaqMan® analysis, and suggest that letrozole induced upregulation of Cyp3a gene expression is as a result of increased gene expression or mRNA stabilization.

Slight increases in POR expression are observed following treatment with letrozole at the highest two doses in males (1.4-1.6-fold). This would be commensurate with the increase in Cyp1a, Cyp2b, Cyp2c and Cyp3a expression, and indicates an increase in cytochrome P450-mediated metabolism. However, these are very small inductions, and therefore the clinical relevance is questionable.

3.3.2.3 Sexual dimorphism in the induction of Cytochrome P450 enzyme activity

In order to dissect which cytochrome P450 activities are induced, a panel of fluorigenic coumarin probes was employed to assay the microsomal extracts previously tested by immunoblotting. The enzyme activity profiles for males and females vary significantly, and there are marked differences in response to each drug **(Figure 3.3.6)**. A noteworthy observation is the increased intra-population variability seen in the female cohort, which complicates analysis.

Examining letrozole induced enzyme activity in males, a significant induction in BFC activity of approximately 2-fold is seen following all doses. An extremely significant increase in BQ activity of 2.5-fold is also observed in response to the highest letrozole dose, indicating an induction of Cyp3a activity. EFC and MFC



Substrate (S)	Enzyme detected
7-benzyloxy-4-trifluoromethyl-coumarin (BFC)	CYP3A4 CYP1A2 CYP2C19 CYP1B1 CYP1A1
7-ethoxy-4-trifluoromethyl-coumarin (EFC)	CYP2B6 CYP2C19 CYP2E1
7-methoxy-4-trifluoromethyl-coumarin (MFC)	CYP2C9 CYP2B6 CYP2E1
7-benzyloxy-quinoline (BQ)	CYP3A4 CYP1A1/2

Figure 3.3.6: Cytochrome P450 activity in wild type C57 BL/6J mice following 3 day treatment with various doses of Letrozole or Anastrozole

*C57 BL/6J mice (n=3 per group) were treated p.o. daily with either 0.9% Saline, Letrozole (25 (A), 50(B) or 100 (C) mg/kg/day) or Anastrozole (0.3 (D), 10 (E) or 20 (F) mg/kg/day) for 3 days. Cytochrome P450 activity in in male (a) and female (b) liver microsomes was assessed using a panel of coumarin probes (human P450 specificity indicated in table). Data is expressed as fold difference ± S.D. Statistics analysed average specific activity following treatment vs vehicle control. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.*

are also modestly induced in the two lowest dose treatment groups, although only EFC activity is significantly induced in the 50mg/kg cohort. Both EFC and MFC activities are extremely significantly induced in response to treatment with 100mg/kg letrozole, indicating an induction of Cyp2b, Cyp2c and Cyp2e enzymes. In females, there is no induction in any coumarin activity as a result of 25mg/kg letrozole treatment. However, in contrast to the males, significant inductions are seen in all four coumarin assays following 50mg/kg treatment, even when considering the greater inter-individual variation evident in the females. Also in contrast to the observations in males, although enzyme activity is induced between 1.6- and 2.6-fold in all four assays following 100mg/kg letrozole treatment, none of these inductions are considered significant, and all appear to be repressed when considered against the data gathered from the 50mg/kg cohort. This indicates that letrozole-induced enzyme activity is dose-limited in females.

The enzyme activity profile in response to anastrozole induction yields very different observations. Modest inductions in EFC and MFC activity of 2.1/2.2-fold and 2.6/2.7-fold were identified in males following 10 and 20mg/kg treatment respectively, indicating some induction of Cyp2b, Cyp2c and Cyp2e enzymes. However, at no dose tested in males does any enzyme activity increase significantly, with no notable induction at all in BFC or BQ activities. In females a very different activity profile is apparent. As in males, no induction is seen with any probe following treatment with 0.3mg/kg anastrozole. However, modest to strong inductions with all probes are seen following treatment with 10mg/kg anastrozole, although only MFC activity is induced significantly. This is probably as a result of the higher inter-individual variation which seems characteristic of the female cohort. This trend suggests that there is induction in a range of cytochrome P450s in response to anastrozole treatment. This finding is given further credence by the data obtained from the 20mg/kg treatment population, in which activity readings for all probes are significantly induced, with inductions in BFC (6.7 ± 1 fold) and MFC (5.8 ± 1.3 fold) activity being extremely significant and induction in EFC (5.5 ± 0.8 fold) being very

significant. The lowest induction in females was seen in the BQ assay (2.2 ± 0.2 fold).

3.4 Discussion

3.4.1 The aromatase inhibitor letrozole is a likely candidate to interact with PXR and CAR

To enable examination of the effects of a range of drugs on a system, we elected to use a methodology employing a panel of mice transgenic for PXR and CAR (Scheer et al., 2008; Ross et al., 2010; Scheer et al., 2010) However, before testing in these models occurred it was essential to identify drugs which were likely to interact with these nuclear receptors to avoid unnecessary experimentation. Initial analysis was performed in C57BL/6J wild-type mice, and used expression and activity of key cytochrome P450s to identify potential interactions. The drugs were selected on the basis that they are all substrates of CYP3A4 (Scripture et al., 2005; Purnapatre et al., 2008), indicating a possible interaction with PXR which prototypically controls this enzyme.

The data from all analyses was striking, with letrozole widely inducing cytochrome P450 expression and activity to significant levels in both males and females. Examining the induction profile following letrozole treatment suggests that it is likely to interact with CAR, although whether directly or indirectly is unclear. *Cyp2b10*, an enzyme prototypically induced by CAR, is upregulated at both the mRNA and protein levels. Further evidence is provided by the upregulation of Cyp1a, Cyp2a and Cyp2c enzymes, all of which have been identified as downstream targets of this nuclear receptor (Ferguson et al., 2002; Wei et al., 2002; Pascussi et al., 2003; Wang and Negishi, 2003; Ferguson et al., 2005; Gillberg et al., 2006; Hernandez et al., 2006; Jackson et al., 2006; Pustylnyak et al., 2007; Chen and Goldstein, 2009; Yoshinari et al., 2010). This conclusion also correlates with the increased enzyme activity observed using the EFC and MFC fluorigenic assays, both of which detect increases in Cyp2b,

Cyp2c and Cyp2e isoforms. On the basis of these data, the role of letrozole as a direct or indirect activator of CAR seems likely.

Letrozole is reportedly metabolised by CYP3A4 and CYP2A6 (Haynes et al., 2003; Tao et al., 2007; Murai et al., 2009), and therefore any modulation of Cyp3a enzyme activity is likely to increase the rate of drug disposal. Interestingly, although *Cyp3a11* mRNA was induced in both sexes, in neither sex did this translate to an induction of Cyp3a protein. However, in males a highly significant increase in activity was seen with those probes associated with Cyp3a, whilst no induction was observed in the females. There could be two explanations for this phenomenon. Firstly, letrozole does not induce Cyp3a activity, but instead increases Cyp1a activity, the minor enzyme detected by BFC and BQ. This hypothesis is given some credence by the strong induction in Cyp1a protein seen on Western blot analysis. However, another explanation is that although overall expression of Cyp3a is not changed, the proportion of active enzyme is increased as a result of increased protein turnover, a finding consistent with data obtained by TaqMan® analysis. It is impossible to identify which hypothesis is correct on the basis of these data, but the use of the more specific resorufin probes in future experiments should provide clarity on this point. It is also important to note that this observation is sexually dimorphic, and therefore any explanation is likely to incorporate gender-specific physiology. Cyp2a is also upregulated at the protein level in males only, indicating that there could be a gender influence in the metabolism in letrozole as a result of the sexually dimorphic expression of Cyp2a and Cyp3a enzymes. This topic will be considered in detail in *Chapter 4*.

Interestingly, both cyclophosphamide and gefitinib induced a similar response, with Cyp1a, Cyp2a and Cyp2b being induced in males on Western blot analysis, and Cyp1a, Cyp2b and Cyp2c being induced in females. Again, the expression profile indicates that both drugs are potential targets of CAR. Cyclophosphamide is known to auto-induce its own metabolism by CYP2B6 and CYP3A4, with the effect being attributed to the agonism of PXR (Chang et al., 1997; Lindley et al., 2002; Harmsen et al., 2009). However, Lindley *et* al. (2002) also noted a more potent induction of CYP2B6 than CYP3A4, suggestive of a role

for CAR. In agreement with these data and our observations, reporter gene assays have recently identified cyclophosphamide as a CAR activator, probably acting via an indirect mechanism, with both PXR and CAR having equal responsibility for auto-induction of cytochrome P450s (Wang et al., 2011). This therefore gives greater confidence that the methodology selected for use in this thesis yields reproducible data.

At this time, there are no reports regarding nuclear receptor activation potential of gefitinib. However, it is known to be primarily metabolised by CYP3A4 in the liver (McKillop et al., 2005; Li et al., 2009a; Duckett and Cameron, 2011) which suggests the possibility of interaction with PXR. It is also metabolised by CYP1A1 in the lungs (Li et al., 2009a; Duckett and Cameron, 2011), an important finding for a medicine recommended by NICE for the treatment of non-small cell lung cancer (http://publications.nice.org.uk/gefitinib-for-the-first-line-treatment-of-locally-advanced-or-metastatic-non-small-cell-lung-cancer-ta192). CYP1A1 is upregulated in the lungs of smokers as a result of activation of the aryl hydrocarbon receptor (AhR). However, recent evidence has also identified CAR as a regulator of CYP1A1 and CYP1A2 in primary human hepatocytes (Yoshinari et al., 2010), and linked the induction of CYP2B6 following treatment with cigarette smoke extract to CAR activation in HepG2 cells adenovirally transduced with hCAR (Washio et al., 2011) although this latter finding needs further evidence from a more suitable model, such as the EYFP-hCAR PHH model discussed in *Section 3.1.1.2*. The expression of CAR in lung tissue is equivocal, with lung specific splice variants located in rats (Kanno et al., 2005a), but no expression identified in humans (Thum et al., 2006). However, the latter study does not analyse the presence of splice variants, and by analysing samples obtained by bronchoalveolar lavage and bronchial biopsy could miss low level expression in specific cell types. If it transpires that CAR splice variants are expressed in the lung and that they are involved in the induction of CYP1A1 in this location, this could represent a novel transcriptional control circuit of key importance when considering drugs targeting smoking-induced lung tumours. Our data suggests that gefitinib is a CAR activator, although further study is

required to confirm this. With CAR being implicated in the control of CYP3A4 and CYP1A1 metabolism, there is potential for CAR-induced auto-induction of gefitinib metabolism. There is also potential for drug interaction caused by activation of CAR by cigarette smoke, thus changing drug pharmacokinetics. Therefore, a full analysis of the role of nuclear receptors in the disposition of this drug could be of key importance to clinical practice.

3.4.2 DOSE OPTIMIZATION OF THE AROMATASE INHIBITORS

On the basis of the data from initial experiments, it was decided to progress letrozole for dose optimization in C57BL/6J wild-type mice prior to testing in the transgenic models. The broad enzyme and activity inductions observed following letrozole treatment suggest that this drug could induce DDIs, and since it is taken on a daily basis over a number of years, it has a broader opportunity to interact with co-medications than many cancer treatments. However, the dose tested was extremely high, and therefore two lower doses, 25 and 50 mg/kg, were analysed for their efficacy in inducing cytochrome P450s. It was also decided to re-examine anastrozole, another aromatase inhibitor of the same class as letrozole. At the dose initially tested, there was no induction in expression or enzyme activity. This dose was selected on the basis of laboratory experience in previous pharmacokinetic experiments. However, it could be too low to induce a response that can be observed by conventional protein analysis after that treatment period, and therefore anastrozole concentration was increased to 10 and 20 mg/kg to enable analysis of cytochrome P450 profile.

It becomes immediately apparent that increasing the dose of anastrozole causes induction of cytochrome P450s, with the profile at higher doses imitating that of letrozole in terms of enzymes induced. Cyp2b is particularly induced in both sexes, in concordance with data obtained by TaqMan® analysis which indicates that *Cyp2b10* mRNA is induced in a dose-dependent manner, with significant increases being seen following treatment with 20mg/kg anastrozole. Similar dose-dependent inductions in EFC and MFC activity are observed, although these only attain significance in the female 20mg/kg cohort. Gender-specific

response is also seen in the induction of Cyp1a and Cyp2a in females and Cyp2c in males. It can therefore be suggested that anastrozole also interacts with CAR, in a similar manner to letrozole. Interestingly, the induction in *Cyp3a11* mRNA in males and females at the highest dose does not translate into an induction in Cyp3a protein, as seen following letrozole treatment. However, it does translate into a significant increase in BQ and BFC activity in the females only, indicating a sexually dimorphic variation in response. An induction in Cyp1a protein is seen in females only, although this does not exceed the 2-fold threshold. This is likely to have a bearing on BFC and BQ activity, being the minor enzyme responsible for metabolism of these probes. As with initial letrozole analysis, whether this increased activity is as a result of Cyp1a induction or increased Cyp3a protein turnover cannot be determined on the basis of these data. However, all data clearly identifies the 20mg/kg anastrozole dose as that most appropriate for future studies.

When examining data from the letrozole dose optimisation, it becomes clear that the 25mg/kg treatment group displays no induction in protein levels for the cytochrome P450s tested in the male cohort, although inductions do occur at the mRNA and enzyme activity levels. Once again, TaqMan® data indicates a significant induction in *Cyp3a11* mRNA, which appears to translate to a corresponding increase in BFC activity. However, because BQ activity is not also induced it must be questioned whether this induction is Cyp3a-related or as a result of inducing another cytochrome P450. Non-significant inductions can also be seen following EFC and MFC analysis, indicating that induction is occurring in those enzymes in terms of activity, although at very low levels that are below the limit of detection for immunoblotting. However, when analysing the same dose in the female cohort, the expression profile varies greatly. Firstly, there is a highly significant induction in *Cyp2b10* mRNA, although other probes are not significantly induced. This is not translated to an equivalent induction in protein, and neither is this associated with an induction in any of the activity assays. Ultimately, the induction levels in the 25mg/kg cohort are not sufficient when compared to the higher doses for use in further studies.

The 50mg/kg letrozole dose appears to give much better induction levels than observed following lower dose treatments. All three genes examined by TaqMan® analysis are induced very significantly, with the greatest induction in *Cyp2b10* being translated to an induction in Cyp2b protein levels in both sexes. Slight induction of Cyp3a protein is also seen in both males and females, although this does not reach the two-fold threshold, indicating that increased gene expression is being translated to enzyme induction. Again, sexual dimorphism is observed in the expression of Cyp2a and Cyp2c, with the former being induced in females, and the latter in males. The effects of protein induction translate through to an induction of enzyme activity which is especially marked in the females. Certainly, induction of both protein and enzyme activity seems to be greater in the females than in the males, although similar inductions in gene expression were seen. This suggests that letrozole has a more significant effect on the female population than the male, and this will require further investigation. However, the levels of protein induction observed using the 50mg/kg dose indicate that this is sufficient to obtain meaningful data in future studies, and therefore should be used in preference to the 100mg/kg dose to obviate potential toxicity issues in the mice.

3.5 Conclusions

Of the drug panel tested, all drugs appear to be potential targets of CAR. There is also likely to be some interaction with PXR, although the induction in *Cyp3a11* mRNA does not translate to an induction in protein expression on Western blot analysis. This is in concordance with data published regarding cyclophosphamide and its role as an activator of both PXR and CAR (Chang et al., 1997; Lindley et al., 2002; Harmsen et al., 2009; Wang et al., 2011). However, the response to letrozole treatment was by far the most significant, and therefore this drug was selected for further study.

Dose optimization studies identified the 50mg/kg letrozole dose as the most appropriate for further analysis. However, technical issues in initial analysis falsely identified the 25mg/kg letrozole dose as sufficient to yield a good

cytochrome P450 induction on Western blot analysis, and therefore this was the dose used in further study. Dosing levels were also analysed for the aromatase inhibitor anastrozole, with the highest 20mg/kg dose being selected for further analysis.

Throughout this analysis it has become evident that there is significant sexual dimorphism in the expression profiles for both drugs. This must be carefully considered in future studies, and therefore both sexes must continue to be used.

CHAPTER 4

CHARACTERIZING THE INTERACTION OF AROMATASE INHIBITORS WITH PXR AND CAR

4.1 INTRODUCTION

4.1.1 AROMATASE AND ITS INHIBITORS

As a result of the data discussed in *Chapter 3*, the drugs letrozole and anastrozole were selected for further investigation into their ability to activate PXR and CAR. Both are third generation, non-steroidal aromatase (CYP19) inhibitors (AI), licensed for the treatment of oestrogen receptor (ER)-positive breast cancer in post-menopausal women **(Table 3.1.1)**. Mechanistically, these drugs competitively inhibit the aromatase enzyme by non-covalent binding to the haem element, thus blocking the substrate binding site (Miller and Dixon, 2002; Miller et al., 2008; Lønning, 2011), although recent studies have also indicated AI-induced transcriptional repression of the *CYP19* gene may occur via the micro-RNA *Let-7f*, providing longer term aromatase suppression (Mackay et al., 2007); (Miller et al., 2012). Aromatase is a key component of the oestrogen biosynthesis pathway, catalysing three consecutive hydroxylation steps in the conversion of C-19 androgens to C-18 oestrogens (Hong et al., 2011), and therefore its inhibition leads to a decrease in oestrogen concentrations. It is primarily expressed in ovarian granulosa cells in pre-menopausal women, but is also expressed in a number of other tissues throughout the body, including muscle, adipose tissue, skin and brain (Garcia-Segura et al., 2003; Bulun et al., 2007; Biegon et al., 2010; Chumsri et al., 2011; Luchetti et al., 2011; Inoue et al., 2012).

Aromatase is essential for the correct physiological functioning of many systems. However, it can become problematic when dealing with oestrogen-dependent cancers in which aromatase over-expression is associated with tumour pathology. The role of aromatase in oestrogen-dependent breast cancer has been particularly well characterized, being over-expressed by undifferentiated adipose fibroblasts recruited to the tumour ball to maintain a localized high oestrogen environment and promote tumour growth (Bulun et al., 2007; Bulun and Simpson, 2008; Ito et al., 2011), thus providing the rationale for the clinical efficacy of the AIs. In addition to their licensed

application in the treatment of various breast cancers, the AIs are also being investigated for use in various off-license indications. These include ovarian and endometrial cancers (Bulun et al., 2007; Ito et al., 2011), endometriosis (Nothnick, 2011; Polyzos et al., 2011; Seal et al., 2011) and selected male and female fertility treatments (Montville et al., 2010; Ben-Haroush et al., 2011; Papanikolaou et al., 2011; Pritts, 2011; Sönmezer et al., 2011); (Cavallini et al., 2011; de Ronde and de Jong, 2011; Saylam et al., 2011; Gregoriou et al., 2012). However, although it is clear that AIs could have potential utility in an increasing number of indications, the evidence base for these off-license applications is not yet sufficient for AI therapy to become a clinical paradigm.

4.1.1.1 LETROZOLE

Pharmacology

Letrozole (Femara®) is indicated for the widest variety of oestrogen-dependent breast cancers of the two AIs analysed in this study (see **Table 3.1.1**), with clinical data indicating that it is superior to tamoxifen as adjuvant treatment for early breast cancer (Crivellari et al., 2008; Colleoni et al., 2011; Regan et al., 2011a; Regan et al., 2011b; Chirgwin et al., 2012), as well as in the treatment of locally advanced and metastatic breast cancer (Ellis and Ma, 2007; Goss, 2007; Mouridsen, 2007). Letrozole is also being extensively tested for various off-label applications, including ovarian and endometrial carcinomas, endometriosis, male and female fertility treatment, hormone-induced growth defects and medical abortion. Pharmacologically, it profoundly suppresses oestrogen production whilst exerting no effect on serum levels of follicle stimulating hormone, luteinizing hormone, thyroid stimulating hormone, cortisol, 17α-hydroxyprogesterone, androstenedione and aldosterone following standard therapeutic doses (Iveson et al., 1993; Lipton et al., 1995).

Letrozole suppresses aromatization to a greater extent than anastrozole, achieving a 98.9% inhibition in activity (Dowsett et al., 1995; Dixon et al., 2008). This equates to a suppression of plasma oestradiol concentrations of 96.6%, oestrone of 99.1% and oestrone sulphate of 99.5% (Geisler et al., 2008).

Importantly, intra-tumoural oestradiol was also suppressed by 97.6%, oestrone by 90.7% and oestrone sulphate by 90.1%. It also indicates that letrozole achieves significantly greater oestrogen suppression than anastrozole, both in the plasma and in tissue (Dixon et al., 2008; Geisler et al., 2008). However, the comparative clinical efficacy of letrozole relative to anastrozole is still being investigated (Monnier, 2010).

Letrozole is considered to be a comparatively safe treatment, with a lower risk of thromboembolic events than tamoxifen. However, the extent of oestrogen suppression is directly responsible for the majority of adverse effects associated with this class of drug. Common side effects of aromatase inhibitor therapy include arthralgia, hot flushes, fatigue, myalgia and gynaecological effects (Gonnelli and Petrioli, 2008; Monnier, 2009; Dent et al., 2011). A higher rate of hypercholesterolaemia has also been associated with long term letrozole treatment, although the evidence base for this finding is conflicting (Pandya and Morris, 2006; Gonnelli and Petrioli, 2008; Monnier, 2009; Zidan et al., 2010). However, possibly the most serious adverse events are those that affect the skeleton, including bone resorption, osteoporosis, and bone pain. Bone resorption is a major concern in aromatase inhibitor therapy, with an increased number of fractures being recorded whilst receiving this treatment as a result of the profound oestrogen suppression induced by these drugs (McCloskey et al., 2007; Eidtmann et al., 2010; Edwards et al., 2011; Nuzzo et al., 2012; Zaman et al., 2012). This is commonly treated by co-administration of bisphosphonates, most notably zoledronic acid, which has itself been recently suggested to repress aromatase activity by inhibiting serine phosphorylation, a post-translational modification essential for aromatase activity, indicating a potential positive functional drug-drug interaction with letrozole (Schech et al., 2012).

Metabolism

The primary excretion mechanism of letrozole in humans is hepatic, being metabolised to a pharmacologically inactive carbinol metabolite, 4,4'-(hydroxymethylene)-dibenzonitrile, by CYP2A6 and CYP3A4 in humans (Sioufi et al., 1997a; Murai et al., 2009; Desta et al., 2011; Precht et al., 2012). This

carbinol metabolite is then glucuronidated prior to excretion, although the enzyme responsible for this reaction has not yet been identified **(Figure 4.1.1)**. Recent studies employing human liver microsomal fractions and recombinant human P450s have yielded important information regarding enzyme kinetics for the formation of the carbinol metabolite (Murai et al., 2009; Desta et al., 2011).

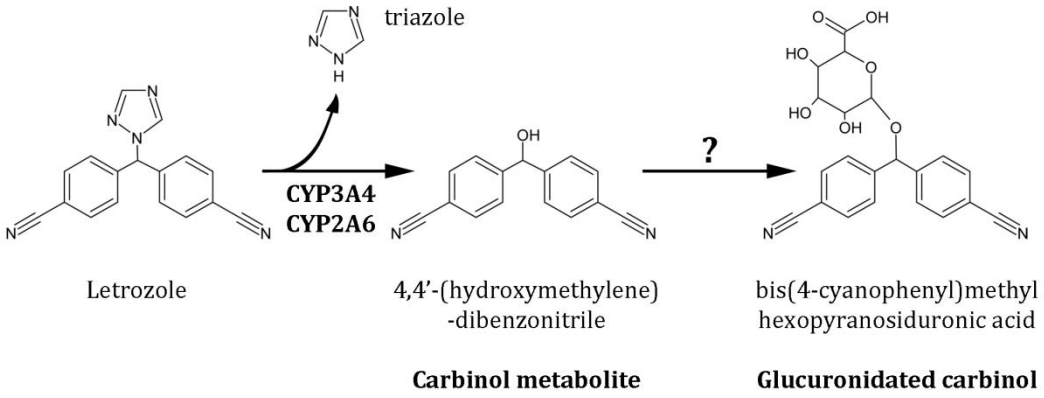


Figure 4.1.1: Proposed metabolism of letrozole in humans *(Sioufi, 1997a; Murai, 2009; Desta, 2011; Precht 2012)*

CYP2A6 has a greater affinity for letrozole than CYP3A4, with K_m of 3.0 ± 1.4 μM and 92 ± 15 μM being calculated respectively (Murai et al., 2009). However, the reaction rate in the CYP3A4 pathway is significantly faster than that of the CYP2A6, having a V_{max} of 22 ± 3 and 4.3 ± 0.2 pmol/min/nmol P450 respectively. The implication of these data is that CYP2A6 is the primary enzyme involved in letrozole metabolism at low concentrations, such as are present during conventional letrozole therapy. If higher doses of letrozole are given, the CYP2A6 pathway becomes saturated, resulting in CYP3A4 becoming the predominant clearance enzyme, and a consequent increase in reaction rate. This cooperative functioning is of particular importance in those individuals possessing CYP2A6 polymorphisms associated with low metabolism, including *CYP2A6*4* (in which the CYP2A6 gene is absent), *CYP2A6*7* and *CYP2A6*9*, all of which are particularly prevalent in Asian populations (≤20% population) (Raunio et al., 2001; Rautio, 2003; Murai et al., 2009; Han et al., 2012).

Pharmacokinetics

Letrozole is absorbed rapidly when given orally, with a bioavailability of 99.9 ± 16.3% being recorded (Sioufi et al., 1997a). The bioavailability does not vary with fed or fasting state, although the rate of uptake is reduced (Sioufi et al., 1997b). However, as a result of the treatment algorithm employed in conventional letrozole therapy, this decrease in absorption rate is unlikely to result in any clinical abnormality and therefore letrozole can be administered regardless of food intake. Plasma protein binding is approximately 60%, mainly to albumin (55.1 ± 1.4%), with concentration in erythrocytes being approximately 80% that of plasma (Colussi et al., 1998).

Letrozole is rapidly distributed within tissues, having a high volume of distribution at steady state (1.87 ± 0.47 L/kg; (Sioufi et al., 1997a)). Interestingly, a recent report examining pharmacokinetics in healthy male subjects (n=52) using a two-compartment model has identified significant association between body fat mass/BMI and peripheral volume of distribution (Jin et al., 2012). They have also found a strong link between body weight and central volume of distribution. This finding concurs with those published by Desta *et al.* (2011), in which letrozole concentrations in postmenopausal women with stage 0-III hormone receptor positive breast cancer (n=284) were found to be negatively correlated with BMI, and positively correlated with age. These findings suggest that body fat composition has a significant impact on the volume of distribution, a hypothesis consistent with the highly lipophilic nature of letrozole.

The pharmacokinetics of letrozole are marginally non-linear in nature at high concentrations, as seen following a high single dose of ≥30 mg or multiple daily doses of 2.5 mg (Pfister et al., 2001). This has been attributed to the saturation of the CYP2A6 pathway at high concentrations, resulting in the CYP3A4 pathway becoming dominant. It is also well documented that letrozole is a strong inhibitor of CYP2A6 activity, thus further decreasing the capacity of this pathway and potentially exacerbating the effect (Pfister et al., 2001; Jeong et al., 2009). Steady-state pharmacokinetics are attained after 2-6 weeks of treatment with 2.5mg letrozole daily.

A summary of published PK parameters in a variety of human populations is shown in **Table 4.1.1**. Although intra-population variation is relatively high as a result of the small scale of these studies, important trends can be dissected from this data. Although there is no variation in C_{max} between healthy male and post-menopausal female populations, there does appear to be a trend towards higher exposure and slower metabolism in the males, with a marginally increased $t_{½}$ and $AUC_{0-\infty}$ than the females. This is likely to be as a result of the higher CYP2A6 activity identified in females (Benowitz et al., 2006). The impact of health status on the pharmacokinetics of letrozole is made clear when comparing the healthy postmenopausal female population with that of the breast cancer population (Sioufi et al., 1997a; Pfister et al., 2001). Changes in pharmacokinetics are observed in all parameters listed in the breast cancer population. T_{max} and $t_{½}$ are both strongly increased, with clearance being decreased. $AUC_{0-\infty}$ is also increased. These findings suggest that metabolism and systemic clearance are inhibited in this population, probably as a result of reduced metabolism, although variation in volume of distribution as a result of physiological changes associated with the disease is also a possibility. It is also worth noting that the intra-population variability in the breast cancer population is much higher than that of the healthy population, most likely as a result of the heterogeneity of this disease.

Another comparison of interest is that of the healthy postmenopausal population against the healthy Japanese population (Sioufi et al., 1997a; Tanii et al., 2011). Although the differences in C_{max} and t_{max} are marginal, it is clear that $t_{½}$, clearance and $AUC_{0-\infty}$ are significantly affected by race. $T_{½}$ and $AUC_{0-\infty}$ are both are both strongly increased in the Japanese population, with clearance being decreased by 70% resulting in a profile that more closely resembles the postmenopausal breast cancer population than the healthy. The enzyme with the highest affinity for letrozole is CYP2A6, and therefore anything that interferes with CYP2A6 activity is likely to have a deleterious effect on letrozole metabolism. It is therefore likely that that higher rate of CYP2A6 genetic polymorphisms in the Japanese population is responsible for the increased systemic exposure observed (Tanii et al., 2011).

Population	Dose	Factors	C_{max}/ nmol/L	t_{max}/ h	$t_{½}$/ h	CL/ L/h	AUC_{0-t}/ nmol h/L	$AUC_{0-\infty}$/ nmol h/L	Reference
Healthy postmenopausal women (n=12)	1	Fasted	130 (approx.)	1.0 (median)	42.0 ± 15.3	2.21 ± 0.65		4290 ± 1471	Sioufi *et al.*, 1997a
Healthy male (n=12)	1	Fasted	129 ± 20.3	0.88 ± 0.38	50.2 ± 21.8		648 ± 73.6	5920 ± 2470	Sioufi *et al.*, 1997b
	1	Fed	98.7 ± 18.6	2.08 ± 1.10	49.7 ± 18.3		591 ± 71.0	5730 ± 2240	
Postmenopausal women with advanced/metastatic breast cancer	1	n=27	107 ± 39.2	8.10 ± 19.0	82.2 ± 55.0	1.52 ± 0.73	1372 ± 541	7387 ± 4063	Pfister *et al.*, 2001
	66	n=24	467 ± 244	3.20 ± 4.91	118 ± 67.4	1.2 ± 0.50	8926 ± 4865		
Healthy Japanese postmenopausal women (n=12)	1	Fasted	151.4 ± 46.4	1.5 ± 0.6	68.6 ± 36.7	1.496 ± 0.702	1959 ± 669	7241 ± 4020	Tanii *et al.*, 2011
	14		652.3 ± 207.1	4.3 ± 2.3	88.9 ± 56.2	0.741 ± 0.265	12999 ± 4143		**Data published as ng/mL. Converted to nmol/L for table*
Healthy Japanese postmenopausal women (n=10)	1		113.6 ± 19.2	2.7 ± 1.2			1629 ± 308		
	28		532.4 ± 185.0	5.5 ± 6.6		0.868 ± 0.334	11555 ± 4444		

Table 4.1.1: Summary of pharmacokinetic data published following 2.5 mg letrozole daily p.o.

Dose indicates length of treatment course in days. Data expressed as mean ± S.D. Source data expressed as CV%, S.D. was calculated using the equation S.D. = mean x CV%. Data from Tanii et al. (2011) converted to nmol/L for ease of comparison.

The effect of these polymorphisms is more pronounced when comparing parameters calculated from the multiple dosing studies (Pfister et al., 2001; Tanii et al., 2011). At steady state, exposure is significantly higher and systemic clearance slower than observed following a single dose in both populations. Systemic exposure at steady-state is much higher in the healthy Japanese population than in the postmenopausal breast cancer group, with C_{max} being increased by 140%, clearance reduced by 62% and AUC_{0-t} increased by 150% (Pfister et al., 2001; Tanii et al., 2011). However, when investigating t_{max} and $t_{½}$ we see that the Japanese population attains the former marginally later, but that $t_{½}$ is 75% faster than in the breast cancer population. It is clear that CYP2A6 genotype is of key importance to letrozole metabolism, and that metabolism in the breast cancer population shares many similarities with a population high in CYP2A6 genetic polymorphisms. However, as reported by Desta *et al.* (2011), CYP2A6 polymorphism alone does not fully account for inter-individual variation in letrozole metabolism, and therefore further investigation of the role of other factors, such as UDP-glucuronosyltransferases and drug transporters, would be of importance for a full analysis of population pharmacokinetics.

Drug interactions

At present, few drug interactions with letrozole have been identified. The most well-documented of these is that of letrozole and tamoxifen. The first study (Dowsett et al., 1999) investigated the impact of tamoxifen on letrozole pharmacokinetics in a postmenopausal locally advanced/locoregional recurrent/metastatic, hormone receptor positive breast cancer population (n=12) after a treatment regime of 6 weeks of letrozole monotherapy (2.5 mg p.o. daily) followed by 6 weeks of letrozole (2.5 mg p.o. daily) + tamoxifen (20 mg p.o. daily). It found that letrozole plasma concentrations and AUC were both significantly reduced following combination therapy, with the latter being reduced by an average of 38%. Unfortunately, the sample size of this study was insufficient to assess the effect on drug efficacy, although the reduction in concentrations and AUC indicates that letrozole dose is effectively decreased to 1.5-2 mg. The changes in pharmacokinetics following combination therapy have been attributed to induction of CYP3A4 by tamoxifen, resulting in

increased letrozole metabolism (Dowsett et al., 1999; Desai et al., 2002; Sane et al., 2008b). A concomitant trial using the same methodology in a postmenopausal progressive metastatic breast cancer population, but in which tamoxifen monotherapy was given for 6 weeks prior to tamoxifen + letrozole combination therapy, indicated that this finding was not reciprocal, with no changes being recorded in the pharmacokinetics of tamoxifen or its metabolites, *N*-desmethyl-tamoxifen or 4-hydroxy-tamoxifen, following combination therapy (Ingle et al., 1999).

Letrozole to date has predominantly been administered as monotherapy in an oncology setting. However, recently there has been increasing interest in novel combination regimes in which letrozole is combined with drugs that target other pathological characteristics of breast cancers, thus increasing the opportunity for adverse drug-drug interactions. These trials are particularly targeted towards the treatment of HER2+ metastatic breast cancer in post-menopausal women, and include the combination of letrozole with trastuzumab, lapatinib and gonadotrophin releasing hormone analogues. The combination of concurrent letrozole + trastuzumab has been found to be more effective than letrozole monotherapy in the treatment of early stage HER2+ metastatic breast cancer (Fleeman et al., 2011; Koeberle et al., 2011; Huober et al., 2012). Lapatinib + letrozole has also been shown to be more effective than letrozole monotherapy in this population (Chu et al., 2008; Sherrill et al., 2010; Fleeman et al., 2011). One study of particular interest combines letrozole with a commonly used neoadjuvant chemotherapy regime (5-fluorouracil 600mg/m^2/ doxorubicin 60mg/m^2/cyclophosphamide 600mg/m^2 administered every 3 weeks) in the treatment of locally advanced breast cancer (Mohammadianpanah et al., 2012). Although this was a relatively small study (n=50 per study arm) and ER status was not known at the time of treatment starting, both clinical and pathological response rates were notably, although not significantly, affected. This was especially prominent once ER status was included in analysis, with a significant increase in overall clinical response of 26% over those women receiving neoadjuvant chemotherapy alone in an ER-positive population (92.74% vs 66.66%). However, no pharmacokinetic

analysis was included in the study design, and this is of key importance when considering the metabolic pathways concerned with metabolising these drugs, and in particular cyclophosphamide which relies on CYP2A6 and CYP2B6 among others.

Although few adverse interactions have been identified when co-administering other drugs alongside letrozole, it is increasingly likely that letrozole will be taken concurrently with various other pharmaceuticals and herbal medicines during breast cancer treatment, as a result of the population age commonly associated with this disease. The novel therapeutic areas and new therapeutic regimes being investigated are also of concern with respect to drug-drug interactions, because of the potential for variability in pharmacokinetics and pharmacodynamics as a result of the altered treatment demographic and increased doses employed. In addition to the lower age of the test populations being investigated for these new applications, the subjects are also in different developmental stages (pre-puberty → post-menopausal) and of different genders, all of which can have significant effects on drug pharmacokinetics. There is also a paucity of good quality pharmacokinetic data associated with these clinical trials, making prediction of potential DDIs problematic. To date there has not been a systematic pharmacokinetic analysis of letrozole with commonly co-prescribed medications, and this becomes increasingly concerning with the higher concentrations employed in areas such as fertility treatment, as well as the increasing role of letrozole as part of a co-administration regime, particularly in the field of oncology in which the therapeutic indices of many drugs are very narrow. In addition, the potential role of letrozole as a PXR or CAR ligand/interactor has not been well defined in the literature, a factor that could significantly impact its potential for DDIs. A full consideration of pharmacology and pharmacokinetics is therefore essential for the continuing assessment of DDI potential.

4.1.1.2 ANASTROZOLE

Pharmacology

Anastrozole (Arimidex®) is a third generation, competitive aromatase inhibitor licensed for use in a number of oestrogen-dependent breast cancers, although it was initially approved for use in advanced breast cancer (see **Table 3.1.**1) (Plourde et al., 1994; Plourde et al., 1995; Buzdar et al., 1997). It has been found to be superior to tamoxifen as adjuvant therapy in early ER-positive breast cancer, giving improved disease free survival, time to recurrence and time to contralateral breast cancer, although overall survival was equivalent between the two drugs (Cuzick et al., 2010; Kelly and Buzdar, 2010). Combination therapies of anastrozole with drugs including goserelin and leuprolide acetate are also showing promise in the treatment of pre-menopausal breast cancer and male breast cancer respectively (Carlson et al., 2010; Cheung et al., 2010). Although not as prominent as letrozole, it is also being investigated for a number of novel indications including endometriosis (Bilotas et al., 2010; Ngô et al., 2010; Nothnick, 2011; Polyzos et al., 2011), gynaecomastia in pubertal boys (Mauras et al., 2009) and functional follicular ovarian cysts in girls with peripheral precocious puberty (Engiz et al., 2009). It has also been assessed for use in fertility treatment, although it was shown to be less effective than clomiphene citrate (Tredway et al., 2011; Tredway and Schertz, 2011).

As previously stated, anastrozole does not suppress oestrogen levels as strongly as letrozole (Dixon et al., 2008). Aromatization has been found to be inhibited by 97% following a 1 mg/day dose of anastrozole (Geisler et al., 1996; Lønning, 1998; Geisler et al., 2002; Geisler and Lonning, 2005), with it being suggested that anastrozole does not fit as tightly in the active site of the enzyme as letrozole (Furet et al., 1993; Mouridsen and Bhatnagar, 2005; Monnier, 2010). Plasma oestrone concentrations were suppressed by 96.3%, oestradiol by 89.9-92.8% and oestrone sulphate by 95.3% (Dixon et al., 2008; Geisler et al., 2008). Oestrogen suppression is less marked in tissue, although still significant, with oestrone being suppressed by 83.4%, oestradiol by 89.0% and oestrone

sulphate by 72.9%. At present, the clinical effect of the differential oestrogen suppression between letrozole and anastrozole has not been fully characterised, although data from the ATAC and BIG 1-98 trials suggests that letrozole has the greater clinical potency (Monnier, 2010; Geisler, 2011).

Although effective at suppressing oestrogens, a recent study has highlighted the high inter-individual variability in the pharmacodynamics of anastrozole, with the range of anastrozole-induced oestrogen modulations encompassing modest but detectable suppression, profound suppression, and also an increase in at least one of the oestrogenic compounds in 4% of subjects (Ingle et al., 2010). This inter-individual variability in pharmacodynamics has been largely attributed to inter-individual variations in drug metabolism. Clearly this variability in pharmacodynamics is likely to have a significant effect on drug efficacy and tolerability, and therefore it must be carefully considered when prescribing anastrozole.

The clinical safety profile of anastrozole is similar to that of letrozole, with the majority of side effects being mild/moderate in intensity and associated with oestrogen suppression. The most commonly reported adverse effects in the ATAC trial were hot flushes (35.7%) and arthraligia (35.6%), the latter of which could be as a result of anastrozole-induced increases in pro-inflammatory cytokines (Jingxuan et al., 2009; Kelly and Buzdar, 2010). There were also more reports of hypercholesterolaemia in the anastrozole treated population than in the tamoxifen population, although there was a similar incidence of cardiovascular effects. Once again, one of the most serious adverse effects following anastrozole treatment was that of bone resorption and osteoporosis, with an incidence of both fractures and osteoporosis of 11% in the anastrozole treated population of the ATAC trial (Cuzick et al., 2010; Eastell et al., 2010; Kelly and Buzdar, 2010). Treatment with oral bisphosphonates, such as ibandronate and risedronate, have been shown to result in an approximately 3% increase in bone mineral density (Markopoulos, 2010; Markopoulos et al., 2010). However, at present there is no evidence of improved cancer-related clinical benefit when combining bisphosphonates, such as is reported for letrozole + zoledronic acid (Markopoulos, 2010; Markopoulos et al., 2010).

Metabolism

Although anastrozole has been clinically available for many years, only recently has its metabolism been characterized in the literature. Hepatic metabolism is the primary route of clearance in humans, with 10% being cleared renally as unchanged parent drug (Sanford and Plosker, 2008). The main metabolites of anastrozole were thought to be triazole (major), anastrozole N-glucuronide, hydroxyanastrozole and glucuronidated hydroxyanastrozole, (Sanford and Plosker, 2008; Ingle et al., 2010; Kamdem et al., 2010; Lazarus and Sun, 2010). However, a recent study by Kamdem *et al.* (2010) was unable to detect triazole both *in vitro* and *in vivo*, and therefore, in common with Sanford *et al.* (2008), they identified hydroxyanastrozole as the major metabolite for this drug. However, *in vivo* free hydroxyanastrozole appears to be short lived, with the majority being detected as the glucuronidated form. The proposed metabolic pathway is shown in **Figure 4.1.2**.

Hydroxyanastrozole is the predominant metabolite of CYP3A4 catalysed anastrozole metabolism, although a minor role is suggested for CYP3A5 (Kamdem et al., 2010). Interestingly, specific inhibition of CYP2C8 and CYP2A6 in human liver microsomes indicated strong repression in anastrozole hydroxylation activity. However, this was not confirmed in expressed P450s, with little or no activity detected in either. Glucuronidation of anastrozole is predominantly catalysed by UGT1A4, with minor activity also detected for UGT2B7 and UGT1A3 (Kamdem et al., 2010; Lazarus and Sun, 2010). To date there is no evidence regarding the specific UGTs associated with hydroxyanastrozole conjugation.

Anastrozole has been shown to inhibit CYP1A2, CYP3A and CYP2C9 *in vitro* (Grimm and Dyroff, 1997). Interestingly, the inhibition profile for both CYP1A2 and CYP3A was biphasic, indicating the presence of 2 linear phases. The result is two apparent K_i values for each enzyme: 8 and 80 μM for CYP1A2, and 10 and 55 μM for CYP3A, with Cornish-Bowden plots indicating that anastrozole inhibition of CYP1A2 is competitive, whilst that of CYP3A is mixed (competitive/non-competitive). The apparent K_i of CYP2C9 was also 10 μM,

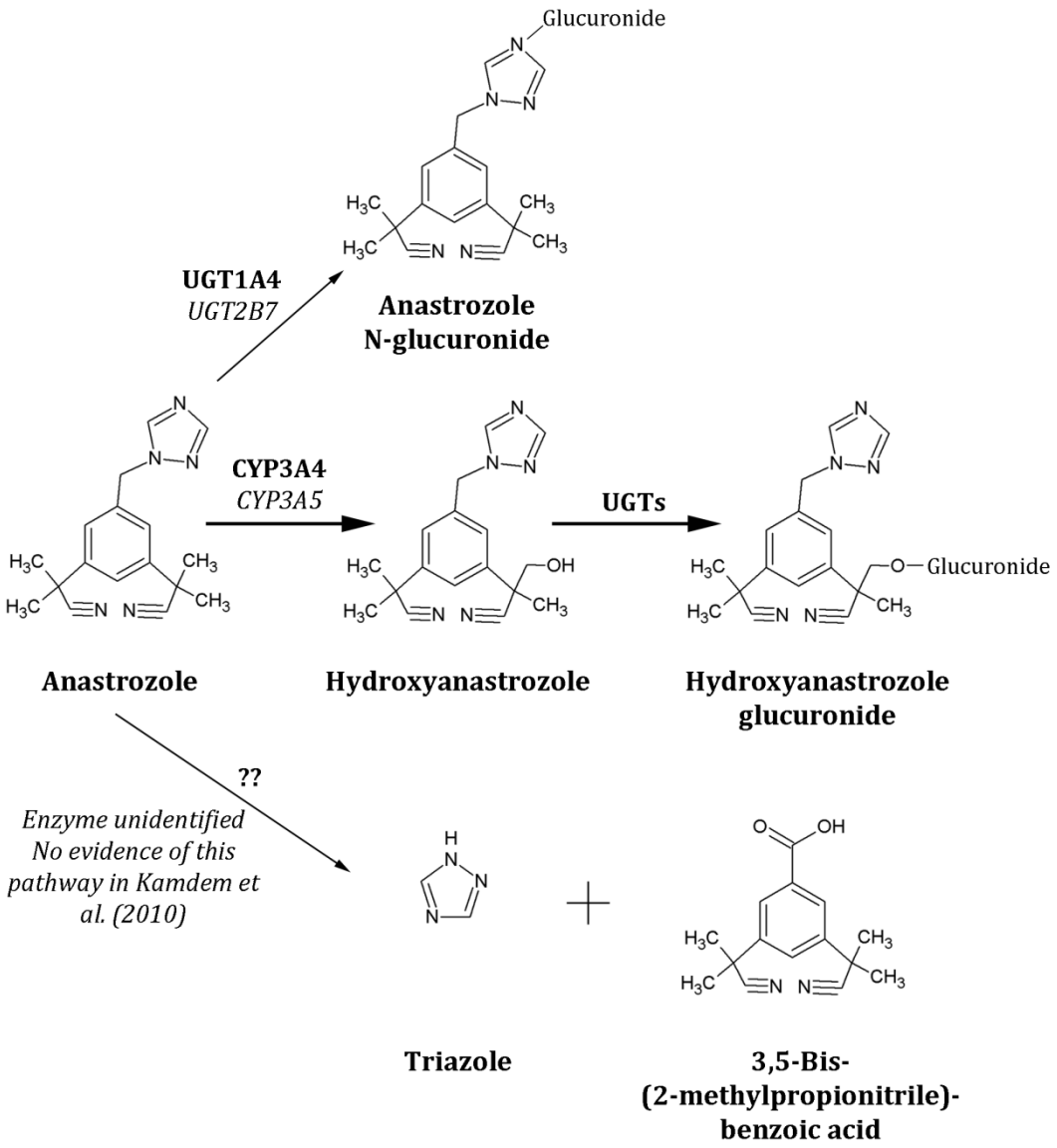


Figure 4.1.2: Proposed metabolism of anastrozole in humans (adapted from Kamdem *et al.* (2010))

with the inhibition being demonstrated to be competitive. Given that the average steady-state C_{max} of anastrozole is 0.3 μM, it is unlikely that any inhibition will occur at standard doses.

There is significant inter-individual variability in the concentrations of anastrozole N-glucuronide, hydroxyanastrozole and hydroxyanastrozole glucuronide (Ingle et al., 2010; Kamdem et al., 2010). An example given in Ingle *et al.* (2010) demonstrates steady state anastrozole concentrations ranging from 0 ng/mL in 2 patients, both of whom possessed measurable anastrozole

metabolite concentrations, to 98.8 ng/mL. Understanding the specific effect a patient's individual enzyme activity profile has on anastrozole pharmacokinetics could significantly improve inter-individual variability in the efficacy and tolerability of anastrozole. It is also important to note that although all anastrozole metabolites are assumed to be inactive, no systematic analysis has been performed to assess the potential activity of these chemicals.

Pharmacokinetics

Analysis of the pharmacokinetics of anastrozole in the literature is problematic as a result of variability and relative paucity of data published. Many early papers do not give detailed PK parameter data, preferring to discuss C_{min} at steady state pharmacokinetics together with changes in oestrogen levels as a measure of drug effectiveness and pharmacokinetic interactions (Plourde et al., 1994; Plourde et al., 1995; Buzdar et al., 1997; Lønning, 1998; Dowsett et al., 2001). It is therefore difficult to obtain a full understanding of anastrozole pharmacokinetics from these initial studies, although basic information is available. Pre-clinical data indicates that anastrozole is rapidly and almost completely absorbed, although these studies have not been published and no quantitative estimate of bioavailability is available (Plourde et al., 1994). However, an early study in healthy Chinese males calculates bioavailability to be 100 ± 9%, confirming this finding (Yuan et al., 2001). Once absorbed, anastrozole is extensively tissue distributed, with a volume of distribution of 98.4 ± 41.9 L (Mauras et al., 2009). Protein binding of anastrozole at therapeutic concentrations is reported to be 40% (Lønning, 1998). Anastrozole pharmacokinetics are linear up to doses of 20 mg/day (Tredway et al., 2004). However, large inter-individual variation in the concentrations of anastrozole and its metabolites has recently been reported, indicating variability in pharmacokinetics (Ingle et al., 2010). Also, a recent study suggests lower efficacy with higher BMI and postulates that this could be a result of an insufficient dose in this population (Sestak et al., 2010). If this is found to be the case, it is likely to be a result of a greater volume of distribution as a result of the higher abundance of adipose tissue.

The most complete pharmacokinetic analysis of early trial data indicates a C_{max} of 13.7 ng/ml, median t_{max} of 2 hours and $t_{½}$ of 40.6 hours following dosing of healthy postmenopausal women (n=7) with 1 mg/day anastrozole (Yates et al., 1996). Steady-state pharmacokinetics are attained following 10 doses of 1 mg/day anastrozole in 90-95% of subjects, with C_{min} of 25.1 ng/ml being recorded at steady state (Plourde et al., 1994; Yates et al., 1996). This is approximately 3.5 fold higher than is recorded after a single dose. Unfortunately no further pharmacokinetic information has been published from the early trials. However, several recent studies have performed full pharmacokinetic analysis in order to validate novel generic formulations of anastrozole in various populations, although none of these allow an analysis of the effect of disease on pharmacokinetics. This data is summarized in **Table 4.1.2**. Certain characteristics can be extracted from this data, although firm conclusions cannot be drawn as a result of the low sample sizes, changes in assay technology and relative paucity of data. Firstly, with the exception of the data generated by Micheal *et al.* (2011), data regarding C_{max} and $t_{½}$ are concordant across populations (Yates et al., 1996; Yuan et al., 2001; Mauras et al., 2009; Noh et al., 2012). The marginally higher concentrations seen in the later studies are most likely as a result of a change in assay technology, with a move from gas chromatography separation with electron capture detection methods (Yates et al., 1996; Yuan et al., 2001) to an HPLC-MS/MS method (Mauras et al., 2009; Micheal et al., 2011; Noh et al., 2012). Although the use of median values precludes the drawing of conclusions with respect to t_{max}, the data does suggest some sexual dimorphism in anastrozole metabolism, with the male populations achieving C_{max} faster than the females. However, within sexes the median t_{max} shows little variation. There also appears to be a potential racial variation in anastrozole pharmacokinetics when examining the data from Micheal *et al.* (2011).

In summary, pharmacokinetic data from anastrozole studies is extremely variable in content, with no systematic analysis of the effects of disease, BMI, race or genetic polymorphisms currently available.

Population	Dosing schedule/ drug	C_{max}/ ng/mL	t_{max}/ h	$t_{½}$/ h	CL/ L/h	AUC_{0-t}/ nmol h/L	$AUC_{0-\infty}$/ nmol h/L	Reference
Healthy postmenopausal women (n=7)	Single Arimidex	13.1	2 (median)	40.6				Yates *et al.* (1996)
Healthy Chinese men (n=20) Fasted	Single Arimidex	10.2 ± 2.5	1.3 ± 0.4	41 ± 26		385 ± 117	429 ± 121	Yuan *et al.* (2001)
	Single Generic	10 ± 3	1.2 ± 0.5	42 ± 14		386 ± 117	443 ± 141	
Healthy postmenopausal/ surgically sterile Indian women (n=14) Fasted	Single Arimidex	16.02 ± 1.59	1.75 (median)	57.06 (median)		800.57 ± 1.66	866.11 ± 1.70	Micheal *et al.* (2011)
	Single Generic	16.24 ± 1.50	1.75 (median)	53.29 (median)		780.11 ± 1.61	834.49 ± 1.64	
Healthy Korean men (n=24) Fasted	Single Arimidex	14.8 ± 2.6	1.0 (median)	42.1 ± 8.4	5.93 ± 5.0%†	681.5 ± 160.5	707.1 ± 177.6	Noh *et al.* (2012)
	Single Generic	15.0 ± 2.3	1.3 (median)	41.6 ± 7.8	3.21 ± 3.8%†	647.8 ± 118.0	669.4 ± 130.0	
Pubertal boys with gynaecomastia (n=42)	14 days Arimidex	39.3 ± 13.5	1.0 (median)	46.8	1.54 ± 0.57	648 ± 240		Mauras *et al.* (2009

Table 4.1.2: Summary of pharmacokinetic data published following 1 mg anastrozole daily p.o.

Dose indicates daily oral dose in mg. Data expressed as mean ± S.D. † = expressed as mean ± relative standard error (standard error as a percentage of mean parameter).

Drug interactions

As in the case of letrozole, information regarding drug-drug interactions with anastrozole is limited, mainly as a result of its primary use as monotherapy. Given the metabolic pathways responsible for anastrozole clearance, any drug that changes the expression and activity of CYP3A4 is likely to result in altered anastrozole pharmacokinetics. This is of particular concern because CYP3A4 is known to be responsible for the metabolism of approximately 60% of currently available drugs, meaning that opportunities for drug-drug interactions are likely to be high (Plant, 2007). However, systematic analysis of potential drug-drug interactions has not been performed. Those interactions that have been analysed are detailed below.

Once again, co-administration of anastrozole with tamoxifen yields a significant decrease in anastrozole concentrations of 27% relative to monotherapy, although there is no effect on those of tamoxifen (Dowsett et al., 2001). Again, it is likely to be as a result of tamoxifen-induced upregulation of CYP3A4 expression via PXR induction (Dowsett et al., 2001; Desai et al., 2002; Sane et al., 2008b), thus increasing metabolism of anastrozole. Although this interaction is unlikely to be of clinical relevance, with oestradiol suppression still reaching the assay limit of detection, it could be of importance in a patient treated with more than one therapeutic that increases the expression and activity of CYP3A4. A recent study has also investigated a possible drug interaction between anastrozole and simvastatin (Bao et al., 2012). Although the group concluded that there was no clinically relevant drug interaction between these two entities, they do report that in 3/9 subjects, a ≥30% change in anastrozole concentrations (2 increased and 1 decreased) was recorded following anastrozole + simvastatin treatment, suggesting that these drugs do interact, probably as a result of simvastatin-induced CYP3A4 expression via PXR induction (Howe et al., 2011). There are no data available regarding the effect of anastrozole on simvastatin pharmacokinetics.

Unfortunately, neither of these studies fully assessed changes in pharmacokinetics, relying instead on the analysis of changes in C_{min}. Although performing the studies once steady state pharmacokinetics are reached does

minimize variation in the PK parameters, it is possible that co-administration alters the pharmacokinetics more substantially than will be evident when purely considering concentration. This means that these studies are likely to miss more subtle changes, such as variations in absorption, AUC and $t_{½}$. Small sample size is also an issue with the study published by Bao *et al.* (2012), in which 1/3 of all subjects demonstrate a ≥30% change and yet pharmacokinetic interactions are dismissed. A larger scale study must be conducted in order to draw any conclusions regarding this interaction. One study that did use a full pharmacokinetic analysis has suggested that anastrozole does not interact with warfarin, although anastrozole pharmacokinetics have not been considered.

Reported drug-drug interactions with anastrozole are therefore rare as a result of the current clinical paradigm. However, as a result of the metabolic pathways involved with anastrozole clearance, together with the growing interest in combination therapies and the prevalent risk of interactions with other prescription and over the counter drugs, it is likely that this could increase in the future. Thorough pharmacokinetic analysis of these combinations, such as are seen in letrozole studies, together with the publication of full pharmacokinetic data from clinical trials are urgently needed in order to assess the risk of clinically relevant drug-drug interactions. It is also of importance to assess the role of anastrozole as a PXR/CAR ligand since this could impact on its own pharmacokinetics as well as on those of other drugs.

4.1.2 THE SPECIES-SPECIFICITY PROBLEM AND TRANSGENIC MODELS

4.1.2.1 SPECIES SPECIFICITY

As discussed in *Chapter 3.1.1.3*, the use of pre-clinical animal models to study pharmacodynamics and pharmacokinetics is common in the pharmaceutical industry because it allows metabolic control and systemic effects to be analysed in a physiologically relevant manner. However, the divergent metabolic control systems present in different animal models limit the potential for extrapolating data to the clinical setting, because they are unable to accurately predict P450

induction in humans (Collins, 2001). With the understanding of the role of nuclear receptors in the control of P450 metabolism has come the realisation that there is also species specificity in their ligand activation, and consequently in the induced expression profile. It is therefore vital to have an understanding of these issues so that the best methodology can be selected for an *in vivo* study.

Species specificity is a complex concept which encompasses all inter-species variations that could affect drug pharmacokinetics and pharmacodynamics, including metabolism, metabolic control, endocrine and neuroendocrine profiles, and body composition. There are many potential causes of species specificity. These include differences in the activity of nucleocytoplasmic shuttling sequences, resulting in aberrant gene expression (Kanno et al., 2005b; Kanno et al., 2007; Kanno and Inouye, 2008), changes in co-factor recruitment, such as SRC-1 interactions (Dau et al., 2012), as well as variations in nuclear receptor response to other signalling pathways (Lichti-Kaiser et al., 2009b). However, a major subset of inter-species variations in drug metabolism occur as a result of differences in the ligand binding domain of the controlling nuclear receptors, such as PXR and CAR, leading to species differential induction of P450s (Jones et al., 2000; LeCluyse, 2001; Östberg et al., 2002; Tirona et al., 2004; Jyrkkärinne et al., 2005; Poso and Honkakoski, 2006; Graham and Lake, 2008; Repo et al., 2008). These receptors show significant species divergence in the amino acid sequence of their ligand binding domains. An examination of variations in amino acid sequence between cloned PXR sequences for rabbit, rat and mouse indicates an amino acid identity of 82.0%, 75.9% and 77.3% respectively in the ligand binding domain relative to human PXR (Jones et al., 2000). This is in contrast to approximately 95% similarity in the DNA binding domain of these species. Other groups have further characterized this variation by identifying the amino acids associated with species specific responses in various species (Östberg et al., 2002; Tirona et al., 2004). Similar divergence is seen in the LBD of CAR, with amino acid identity between mouse and human CAR being reported at approximately 70% in this region (Baes et al., 1994; Choi et al., 1997; Moore et al., 2000; di Masi et al., 2009). The significant divergence

in sequence in the ligand binding domain across species results in variations in ligand binding and activation potential of

Drug	Interacts with	Species			
		Mouse		*Human*	
		EC_{50} (nM)	*Action*	*EC_{50} (nM)*	*Action*
PCN*	**PXR**	200-700	+	>10,000	N/A
Rifampicin*		NR		200-3000	+
SR12813		4100	+	120-200	+
Hyperforin		NR		23	+
Schisandrin		1250	+	2000	+
5β-Cholestan-3α,7α,12α-triol		2500	+	5000	+
TCPOBOP*	**CAR**	20-100	+	No effect	N/A
CITCO*		NR		25-304	+
5α-Androstan-3α-ol		250-1500	-	1000->10000	-
Meclizine		25	+	500-1000	-
Clotrimazole		No effect		50-1000	-
5β-Pregnane-3,20-dione		670-3000	+		Weak
				>10,000	+
				>>10,000	-

Table 4.1.3: PXR/CAR ligands which demonstrate species specific activity

NR = not reported. + = agonist, - = antagonist (inverse agonist, CAR only). Table adapted from Fraser et al. *(2012). PXR and CAR data adapted from di Masi et al. (2009), Stanley et al. (2006) and Moore et al. (2000). * indicates prototypical inducer.*

various chemicals with respect to PXR and CAR, and is a major cause of interspecies variation in drug metabolism (Moore et al., 2000; di Masi et al., 2009). The importance of species specific nuclear receptor activation to pre-clinical research is highlighted by a number of drugs which demonstrate differential activation of PXR and CAR between species, a selection of which are described in **Table 4.1.3**.

In addition to providing a useful selection of tools for nuclear receptor studies, the identification of inter-species variation also highlights a potentially serious limitation of the use of animal models for pre-clinical studies, namely that of

species specific nuclear receptor activation. As demonstrated in **Table 4.1.3**, the nuclear receptor activation potential of a chemical can vary widely between homologues. Failure to fully consider these variations prior to clinical trials could therefore result in the clinical failure of a drug, serious adverse events and severe toxicity as a direct result of differential nuclear receptor activation and incorrect inter-species extrapolation. A number of transgenic *in vivo* models have therefore been specifically engineered to address this problem.

4.1.2.2 TRANSGENIC MODELS

The use of transgenic models, commonly mouse models in which a gene of interest is modified, is a cornerstone of *in vivo* analysis. With correct design and selection, these models allow the role of selected genes to be characterized in a physiologically relevant setting in which data regarding the downstream effects of gene modification, such as target gene expression and pharmacokinetics, can also be analysed. One setting in which these models have demonstrated particular efficacy is in the analysis of nuclear receptor activation. In order to allow a more physiologically relevant analysis of PXR/CAR activation profiles, numerous transgenic mouse models have been derived in which one or both genes are knocked-out (KO) or replaced with the human gene (humanized) (Xie et al., 2000; Staudinger et al., 2001; Wei et al., 2002; Zhang et al., 2002; Saini et al., 2004; Huang et al., 2005; Hernandez et al., 2007; Ma et al., 2007; Cheung and Gonzalez, 2008; Lichti-Kaiser and Staudinger, 2008; Ma et al., 2008a; Scheer et al., 2008; Ross et al., 2010; Scheer et al., 2010; Cheng et al., 2011; Hasegawa et al., 2011). Collectively, these models allow ligand interactions with the PXR/CAR signalling pathways to be dissected at the same time as providing meaningful data regarding the downstream effects of activation. They also have the advantage that mouse lines carrying the modification of interest can be continuously bred without the loss of the transgenic genes, as well as the potential for deriving more complex multiple transgenic models through selective cross-breeding.

There are various approaches to the construction of these transgenic models (Xie et al., 2000; Zhang et al., 2002; Saini et al., 2004; Huang et al., 2005; Hernandez et al., 2007; Lichti-Kaiser and Staudinger, 2008; Mota et al., 2011). However, the construction of the models selected for this study, developed and provided by CXR Biosciences Ltd. and TaconicArtemis, employed a targeted knock-in strategy, described in **Figure 4.1.3 & 4.1.4**, in which human PXR/CAR are inserted into the corresponding mouse locus under the control of the innate murine promoter, and in such a way as to disrupt the murine gene (Scheer et al., 2008; Ross et al., 2010; Scheer et al., 2010). The advantage of this method is that by employing the innate murine promoter all necessary transcription co-factors, enhancers, etc. are available. This ensures that the global expression pattern and magnitude in this model are comparable to those of wild-type mice, as well as showing equivalence to those found in human liver samples (Scheer et al., 2008).

This model panel not only allows the analysis of ligand interactions with PXR and CAR, but also in depth analysis of the regulatory crosstalk between these two nuclear receptors in target gene regulation and of the implications of species specific interactions on downstream gene expression. However, transcriptional regulation of human PXR/CAR themselves cannot be assessed in this model because the transgene is under the control of the murine promoter. The design of these models also allows the production of PXR/CAR splice variants, an increasingly important factor in assessing inter-individual variation and response to therapies. Three hCAR splice variants (hCAR.1, hCAR.2 and hCAR.3) have been identified in the HuCAR models, in which the full sequence of introns and exons from exons 2-9 of the hCAR gene was inserted (Scheer et al., 2008). Three PXR splice variants (hPXR.1, hPXR.2 and hPXR.3) have been identified in the HuPXR models, in which the transgene comprises a fusion of hPXR exons 2-4, full genomic sequence between exons 4-8 and a fusion of exon 8-9 (Scheer et al., 2008; Scheer et al., 2010). Subsequent analysis of a derived PXR/CAR double humanized model has identified detectable levels of all splice variants, with proportionate expression being comparable to that of human

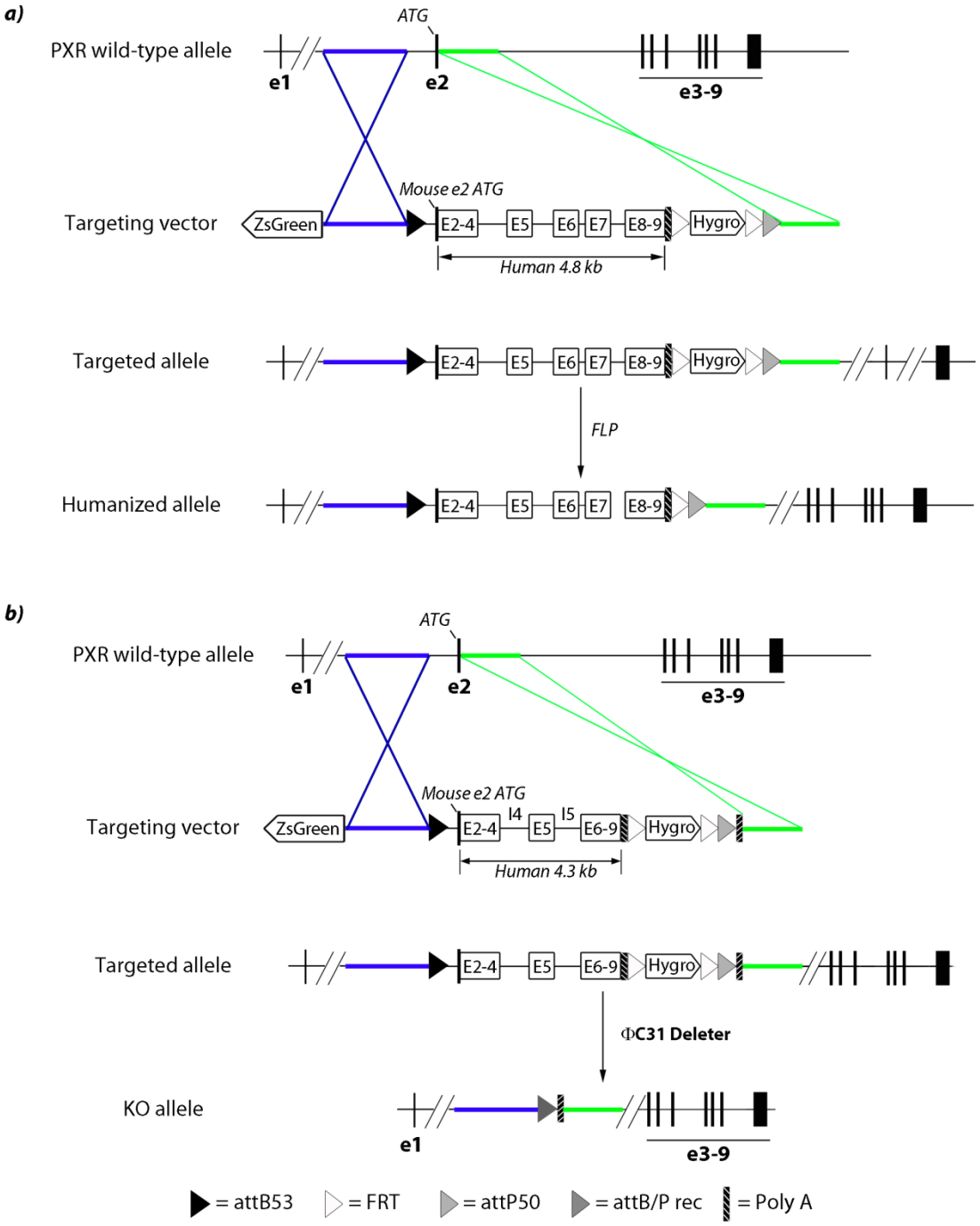


Figure 4.1.3: Construction strategy of HuPXR (a) and PXR KO (b) mice (Adapted from Scheer *et al.* (2008) & Scheer *et al.* (2010))

a) *HuPXR model (Scheer* et al.*(2010)): hPXR minigene consisting of a fusion of human exons 2-4, full genomic sequence from exons 4-8 and a fusion of exons 8-9 was knocked into the murine wild-type ATG transcriptional start site, disrupting the native mPXR gene. Mice were crossed onto a strain expressing FLPe recombinase to excise the hygromycin selection cassette through FLP-mediated recombination at FRT sites. b) PXR KO model (Scheer* et al. *(2008)): Derived from old PXR model in which hPXR minigene containing a fusion of exons 2-4, genomic sequence from exons 4-6 and a fusion of exons 6-9. Mice were crossed onto a ΦC31 deleter strain to generate PXR KO model. Black exons + lower case label = murine, white exons + upper case label = human.*

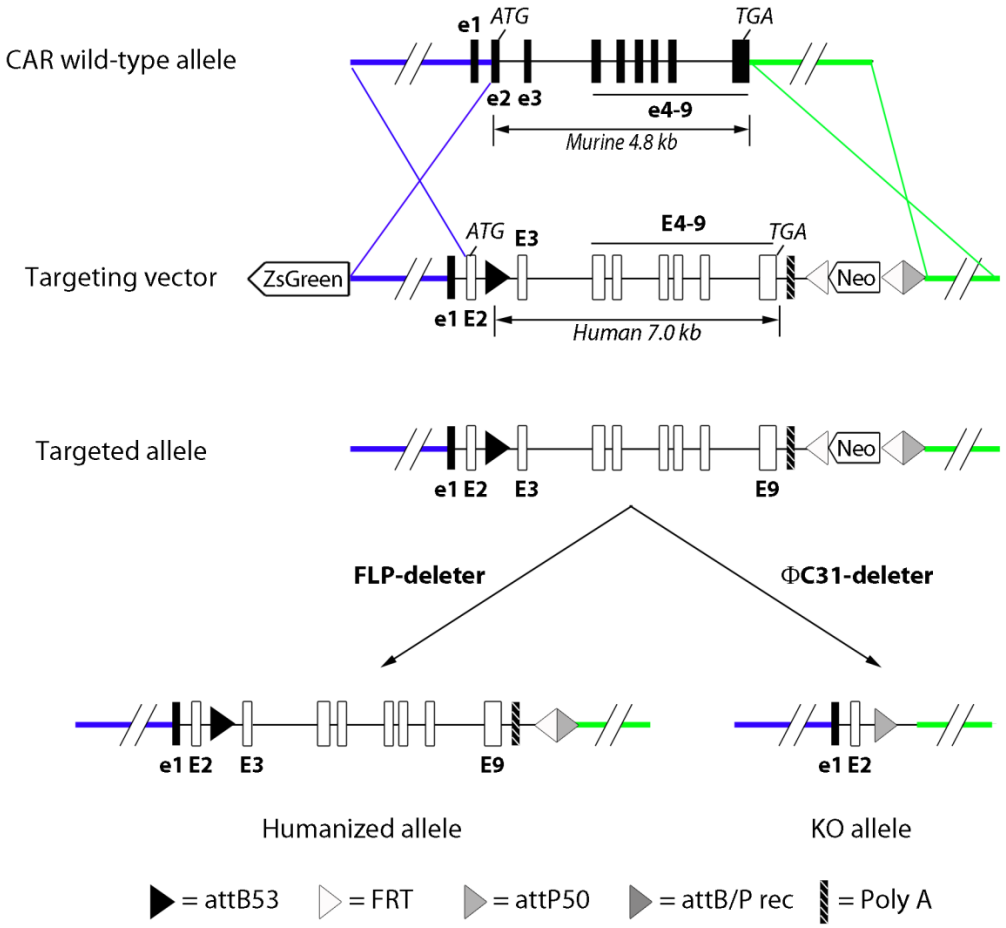


Figure 4.1.4: Construction strategy of HuCAR and CAR KO mice (Adapted from Scheer *et al.* (2008))

Murine CAR coding region (exons 2-9) replaced with the human CAR coding region (exons 2-9). Mice were crossed onto an FLPe-recombinase expressing strain to generate the HuCAR model through FLP-mediated recombination at the FRT sites, removing the neomycin selection cassette. CAR KO models were derived by crossing mice onto a ΦC31 deleter strain. Black exons + lower case label = murine, white exons + upper case label = human.

liver tissue, with the exception of hPXR.2 which was four-fold greater in the transgenic model (Ross et al., 2010).

Another useful class of transgenic models for the analysis of drug metabolism are those in which certain drug metabolising enzymes are humanized. The advantage of these models is that species-specificity with respect to enzyme function can be analysed, thus potentially yielding more clinically relevant data. These models can also provide important information regarding differential gene regulation between species. Numerous models are available which

express human P450s, notably CYP1A1/1A2, CYP2D6, CYP2E1 and CYP3A4 (Van Herwaarden et al., 2007; Cheung and Gonzalez, 2008; Scheer et al., 2010; Boverhof et al., 2011; Cheng et al., 2011; Hasegawa et al., 2011; Levova et al., 2012; Scheer et al., 2012). Many of these have been selectively bred onto a humanized PXR/CAR background in order to provide models which give a more relevant analysis of human P450 transcriptional regulation (Ma et al., 2008a; Hasegawa et al., 2011). However, alongside the development of these models, a number of reporter lines in which a section of the 5' upstream regulatory region of a P450 gene is linked to the start codon of a reporter gene, such as *lacZ* (Robertson et al., 2003; Stedman et al., 2004; Campbell et al., 2005; Charles et al., 2006) or *luciferase* (Zhang et al., 2003), have been developed. These models provide a simple method of analysing cell- and tissue-specific expression of the human gene, and are also useful tools for assessing transcriptional regulation of the reporter-linked gene.

One such model is the novel rCYP2B6-lacZ reporter mouse used in this study, in which approximately 70kb of the 5' regulatory region is linked to a lacZ reporter gene, and randomly inserted into the murine host (developed by TaconicArtemis/CXR Biosciences Ltd., unpublished). The large 5' regulatory region inserted into this model is of sufficient size to incorporate the known key regulatory elements associated with CYP2B6 expression, including the PBREM and distal XREM (Wang and Tompkins, 2008; Inoue and Negishi, 2009; Benet et al., 2010b; Fraser et al., 2012). This construct is available on both mPXR/mCAR and hPXR/hCAR backgrounds, and therefore allows the analysis of differential regulation of the CYP2B6 gene by the murine and human receptors. In *Chapter 3*, one of the features of aromatase inhibitor-induced P450 expression was induction of Cyp2b10, the mouse orthologue of human CYP2B6 which shares many of its regulatory motifs (*See Section 1.2.3.1*). The ability of these drugs to induce this enzyme is of clinical concern, because it has a key role in the metabolism of many clinically important drugs, including drugs used in oncological treatment regimes, such as ifosfamide and tamoxifen (Wang and Tompkins, 2008). Of particular concern is the pro-drug cyclophosphamide, which requires CYP2B6 metabolism to produce its active moiety, and which is a

key component of many chemotherapeutic regimes employed in the treatment of breast cancer (Wang and Tompkins, 2008). Any modulation of CYP2B6 induced by the aromatase inhibitors could therefore have significant downstream effects on the efficacy and toxicity of subsequent treatments. The rCYP2B6-lacZ models are therefore a useful tool for the analysis of PXR/CAR mediated induction of CYP2B6 in response the aromatase inhibitors.

4.1.3 AIMS AND OBJECTIVES

In chapter 3, it was demonstrated that the AIs letrozole and anastrozole induced P450 metabolism in a manner suggestive of PXR and CAR activation. In this chapter, the role of PXR and CAR in the P450-mediated metabolism and pharmacokinetics of these drugs will be further characterized using a panel of novel transgenic mouse models **(Figure 4.1.3 & 4.1.4; Table 4.2.1)** (Scheer et al., 2008; Ross et al., 2010; Scheer et al., 2010). Pharmacokinetic, protein expression and activity data will be analysed across all genotypes. Temporal changes in pharmacokinetics will also be investigated. Both drugs will be analysed in males and females to provide data regarding gender-specific variation in nuclear receptor activation, drug metabolism and pharmacokinetics. This methodology will allow the role of nuclear receptor crosstalk to be dissected, as well as an analysis of the impact of species and gender specificity on the metabolism of the aromatase inhibitors. Finally, the impact of the aromatase inhibitors on the expression of CYP2B6 will be assessed in males and females using the rCYP2B6-lacZ reporter line on both the mouse and human PXR/CAR backgrounds. This will allow species-differential induction to be assessed and provide evidence for any potential drug-drug interactions mediated by CYP2B6 in both species. The null hypothesis is that the AIs interact with PXR/CAR in a species- and gender-specific manner to promote drug metabolism via the P450 enzyme system.

4.2 METHODS

4.2.1 PXR AND CAR TRANSGENIC MODEL PHARMACOKINETIC STUDIES

A panel of novel PXR and CAR transgenic mouse models (see **Table 4.2.1**) and a C57 BL/6J wild-type control model (n=3 per group; male and female) were treated *p.o.* on a daily basis for either 1 or 3 days with anastrozole (20 mg/kg), letrozole (25 mg/kg) or an equivalent volume of 0.9% saline vehicle (control). All drug treated animals underwent a pharmacokinetic study following the final drug dose as described in *Section 2.2.3.2*. Following the final timepoint at 24 hours, mice were euthanized using rising CO_2.

Model Name	Notes	Supplied by	Reference
PXR KO	• All models produced by disrupting murine genes. • mPXR exon 2 removed in HuPXR and PXR KO models. • mCAR exons 2-9 removed in HuCAR and CAR KO models. • HuPXR/HuCAR are under control of the mouse promoter	TaconicArtemis/ CXR Biosciences Ltd	Scheer *et al.* (2008)
CAR KO			
HuPXR			
HuCAR			
HuPXR/CAR KO			
PXR KO/HuCAR			
PXR/CAR DKO			
PXR/CAR DHu			
rCYP2B6_lacZ (mPXR/CAR)	• Random insertion of lacZ under the control of ≈ 70kb of the human CYP2B6 promoter. • Model designed on mouse and human PXR/CAR background		CXR Biosciences (unpublished)
rCYP2B6_lacZ (hPXR/CAR)			

Table 4.2.1: Transgenic mouse models used in *in vivo* studies

Following euthanasia, blood was harvested by cardiac puncture into heparinized tubes which were subsequently centrifuged at 16,000 *x g* for 10

minutes. Plasma supernatant was removed to a clean eppendorf which was snap frozen in liquid N_2 and stored at -70˚C prior to analysis. Liver was weighed, then one small piece of liver was harvested into an eppendorf tube, with the remainder being bisected and transferred into bijou tubes. All liver samples were snap frozen in liquid N_2 and stored at -70˚C prior to analysis.

4.2.2 CYP2B6_LACZ TRANSGENIC REPORTER MODEL STUDIES

rCYP2B6_lacZ (mPXR/mCAR background; male and female) and rCYP2B6_lacZ (HuPXR/HuCAR background; male only) transgenic models (see Table 2.1; n=3 per group) were treated p.o. on a daily basis for 3 days with anastrozole (20 mg/kg), letrozole (25 mg/kg) or an equivalent volume of 0.9% saline vehicle control. Mice were euthanized using rising CO_2 24 hours following final dose.

Following euthanasia, blood was harvested by cardiac puncture into heparinized tubes which were subsequently centrifuged at 16,000 *x g* for 10 minutes. Plasma supernatant was removed to a clean eppendorf which was snap frozen in liquid N_2 and stored at -70˚C prior to analysis. Liver was weighed, then lobe 1 was bisected, with one half being fixed in 1% PFA (for cryofixing) and the other half being fixed in GURR (formal saline) (for wax embedding) at 4˚C. After 4 hours, the 1% PFA fixed samples were rinsed twice with PBS (Oxoid, UK) before transferring to 30% sucrose solution at 4˚C overnight, prior to cryofixing the following day and storage at -70˚C. The GURR (formal saline) samples were left at 4˚C overnight, then rinsed twice in PBS and transferred to 70% ethanol for storage at 4˚C prior to wax embedding. Of the remaining liver, one small piece of liver was harvested into an eppendorf tube, with the remainder being bisected and transferred to bijou tubes. All remaining liver samples were snap frozen in liquid N_2 and stored at -70˚C prior to analysis.

4.3 RESULTS

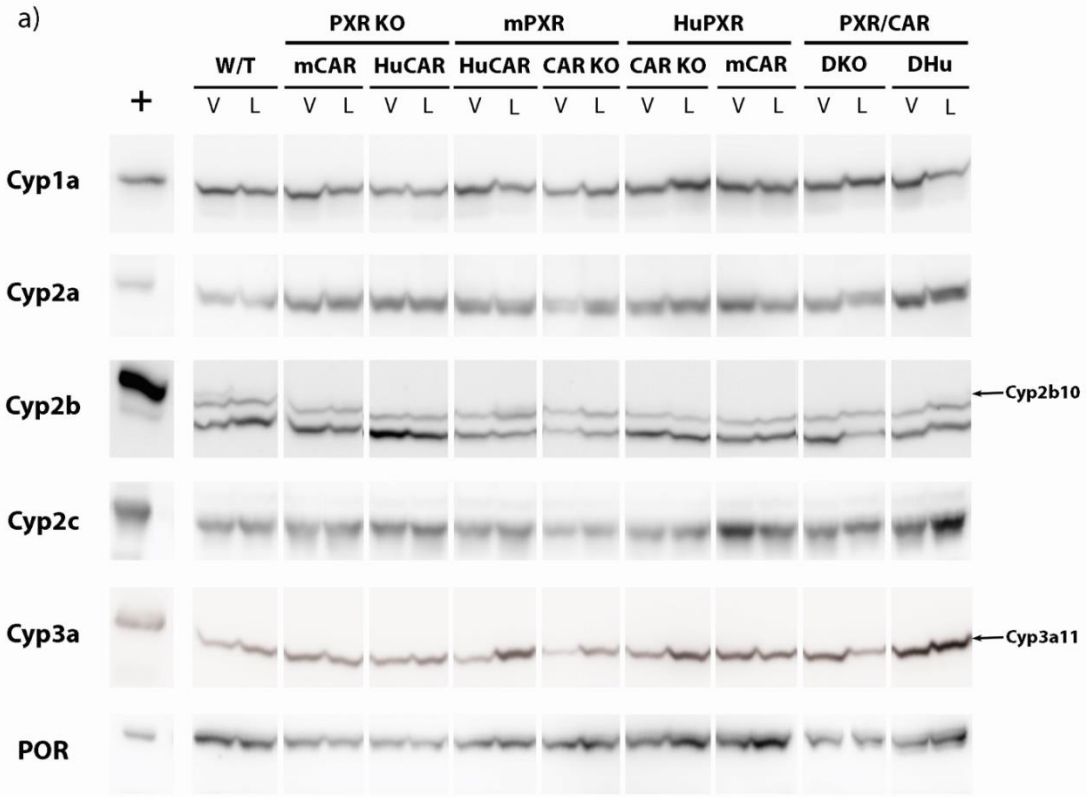
4.3.1 CYTOCHROME P450 INDUCTION IS MEDIATED BY PXR AND CAR

4.3.1.1 LETROZOLE

Western blot analysis indicates that PXR/CAR status is an important factor in the control of expression of certain cytochrome P450s following letrozole treatment. Sexual dimorphism in the expression of certain cytochrome P450s is also a feature, particularly when analysing Cyp2b10 expression. No induction in Cyp1a is seen in any of the genotypes following letrozole treatment in males **(Figure 4.3.1)**, with a similar pattern observed in females **(Figure 4.3.2)**. With respect to Cyp2a, no induction is seen in males or females. No significant induction in Cyp2c is observed in both sexes.

Unsurprisingly, the greatest inductions are seen in Cyp2b and Cyp3a protein, the two enzymes prototypically induced by CAR and PXR activation respectively. Interestingly, there is obvious sexual dimorphism in the expression of Cyp2b10, with no expression seen in males, but basal expression and enzyme induction seen in females. The first feature that is evident in females is that basal and induced expression of Cyp2b10 are absent in all models in which CAR is knocked out (mPXR/CAR KO, HuPXR/CAR KO and PXR/CAR DKO). This concurs with CAR's role as the primary regulator of this enzyme. No induction is seen in the wild-type, PXR KO/HuCAR and HuPXR/mCAR models, although basal expression is observed. It is also interesting that in those models in which PXR has been humanized, but still possessing a functional CAR moiety (HuPXR/mCAR and PXR/CAR DHu) the basal expression of Cyp2b10 is marginally increased, suggesting that HuPXR does not function identically to its murine counterpart. Induction is seen in the PXR KO/mCAR (1.6-fold), mPXR/HuCAR (2.3-fold) and PXR/CAR DHu (1.9-fold) models.

a)

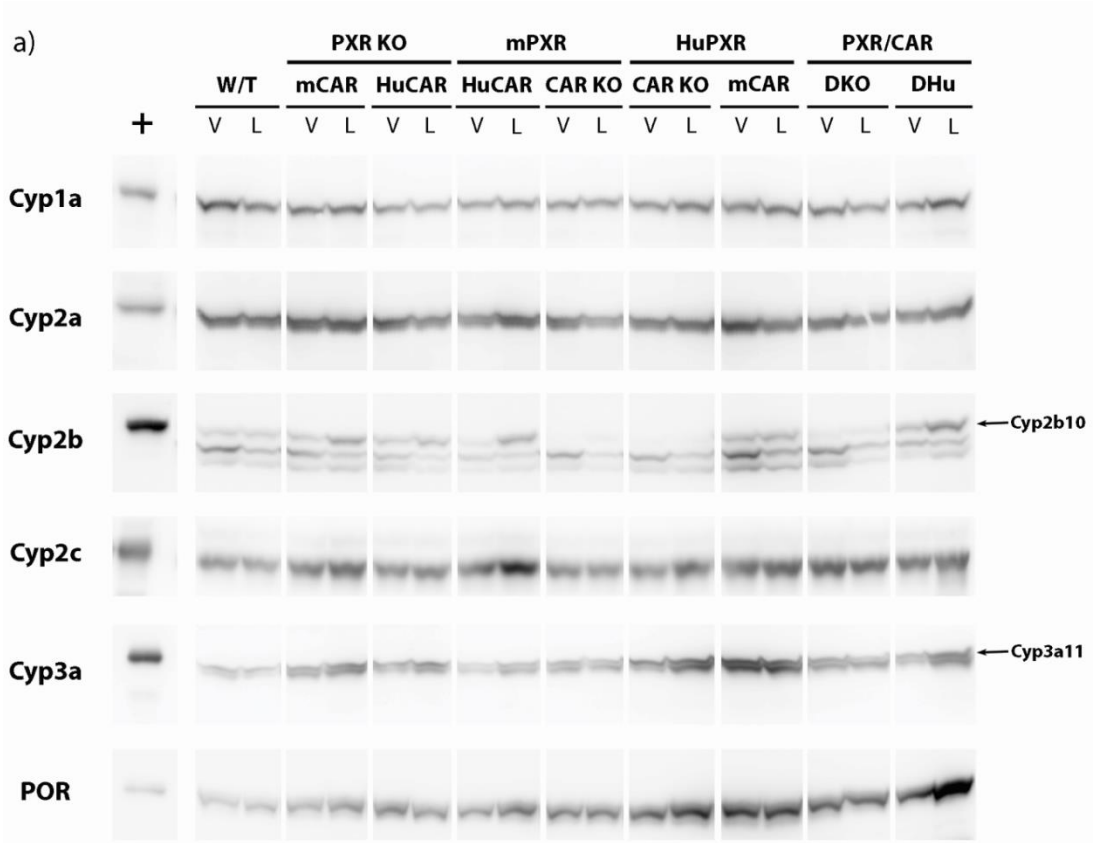


b)

Gene	Genotype								
	W/T	PXR KO		mPXR		HuPXR		PXR/CAR	
		mCAR	HuCAR	HuCAR	CAR KO	CAR KO	mCAR	DKO	DHu
Cyp1a	0.9	0.9	1.0	0.7	1.4	1.3	0.9	1.1	0.6
Cyp2a	1.1	1.1	1.0	0.9	1.5	1.2	0.8	0.9	1.1
Cyp2b	0.9	0.9	1.4	0.9	1.0	1.0	1.0	1.2	0.8
Cyp2c	1.1	1.2	1.1	1.1	1.1	1.3	0.7	1.1	1.5
Cyp3a	1.5	0.9	1.2	2.4	1.8	1.7	0.8	0.6	1.2
POR	0.9	0.8	1.1	1.3	1.2	1.4	1.3	1.0	1.6

Figure 4.3.1: Induction of Cytochrome P450s in male wild type C57BL/6J and transgenic mouse models following 3 day treatment with 25 mg/kg Letrozole

C57BL/6J mice and a panel of PXR and CAR transgenic models (n=3 per group) were treated p.o. daily with either 25 mg/kg Letrozole (L) or 0.9% Saline vehicle control (V) for 3 days. a) Western blots of pooled liver microsomes (n = 3 per group; 20μg/well). + = positive control - Liver microsomes from male C57BL/6J mouse treated with either phenobarbital (80 mg/kg/day for 3 days; 7.5μg/well; Cyp1a, 2a, 2b, and 2c) or dexamethasone (50 mg/kg/day for 3 days; 5μg/well; Cyp3a and POR). b) Densitometry analysis - Fold induction/repression following drug treatment relative to saline control. Numbers in red indicate >2 fold induction. Densitometry is isoform specific for Cyp2b10 and Cyp3a11 only. All other data represent densitometry for the full gene cluster.



b)

Gene	Genotype								
	W/T	PXR KO		mPXR		HuPXR		PXR/CAR	
		mCAR	HuCAR	HuCAR	CAR KO	CAR KO	mCAR	DKO	DHu
Cyp1a	0.7	1.1	0.9	1.2	1.0	1.3	1.0	0.8	1.5
Cyp2a	1.0	1.0	0.8	1.4	0.7	1.2	0.7	0.7	1.3
Cyp2b	1.0	1.6	1.1	2.3	1.0	1.3	1.1	0.9	1.9
Cyp2c	0.8	1.3	1.1	1.6	0.9	1.3	1.2	0.9	1.1
Cyp3a	0.8	1.4	1.3	1.6	0.9	1.7	0.9	0.8	1.9
POR	1.1	1.3	0.8	1.5	1.0	1.5	1.0	0.9	2.0

Figure 4.3.2: Induction of Cytochrome P450s in female wild type C57BL/6J and transgenic mouse models following 3 day treatment with 25 mg/kg Letrozole

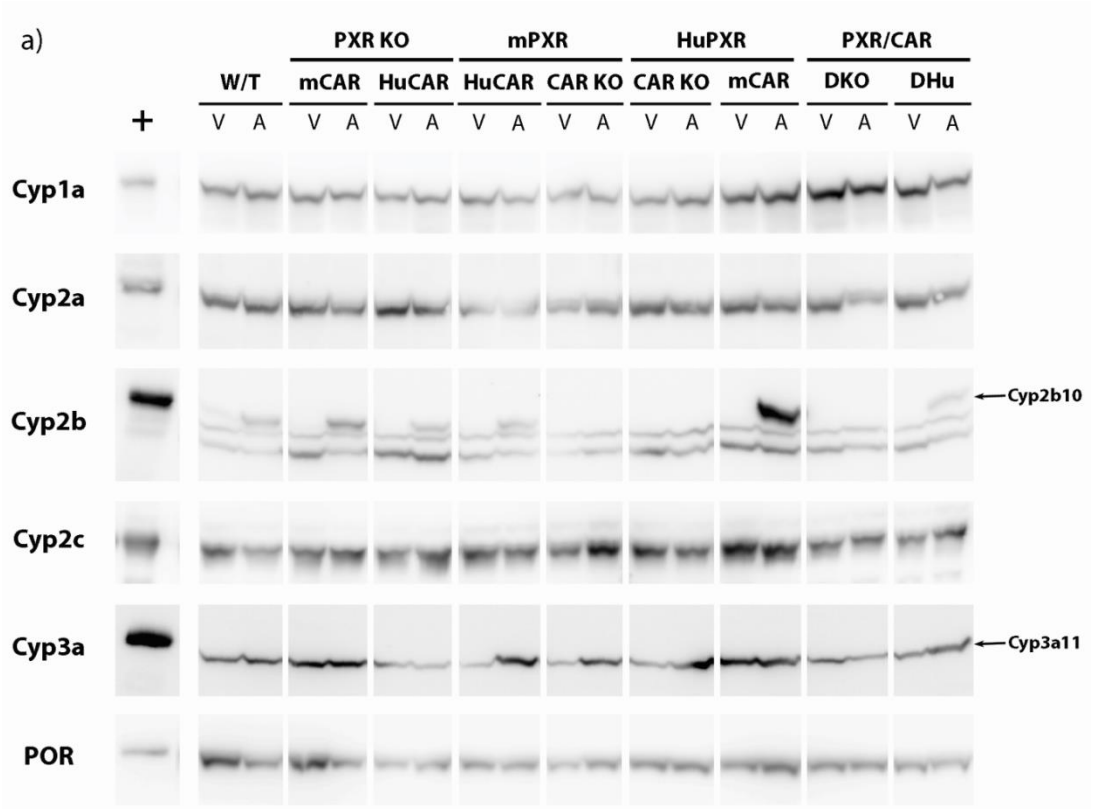
C57BL/6J mice and a panel of PXR and CAR transgenic models (n=3 per group) were treated p.o. daily with either 25 mg/kg Letrozole (L) or 0.9% Saline vehicle control (V) for 3 days. a) Western blots of pooled liver microsomes (n = 3 per group; 20μg/well). + = positive control - Liver microsomes from male C57BL/6J mouse treated with either phenobarbital (80 mg/kg/day for 3 days; 7.5μg/well; Cyp1a, 2a, 2b, and 2c) or dexamethasone (50 mg/kg/day for 3 days; 5μg/well; Cyp3a and POR). b) Densitometry analysis - Fold induction/repression following drug treatment relative to saline control. Numbers in red indicate >2 fold induction. Densitometry is isoform specific for Cyp2b10 and Cyp3a11 only. All other data represent densitometry for the full gene cluster

Unlike Cyp2b10, Cyp3a11 is expressed in both sexes and all genotypes, indicating its greater importance in drug metabolism. Although basal expression is observed, there is no induction in the PXR KO genotypes (PXR KO/mCAR and PXR KO/HuCAR) in either sex, with a slight repression being seen in the PXR/CAR DKO model in males following letrozole treatment. This can be attributed to PXR's key role in Cyp3a11 regulation. In males, induction is seen in all models wild-type for PXR (wild-type [1.5-fold], mPXR/HuCAR [2.4-fold], mPXR/CAR KO [1.8-fold]) and also in the HuPXR/CAR KO models (1.7-fold). No induction is seen in the HuPXR/mCAR or PXR/CAR DHu models. In females, no induction is seen in wild-type, PXR KO models, mPXR/CAR KO, HuPXR/mCAR or PXR/CAR DKO models. Highest induction is found in the PXR/CAR DHu model (1.9-fold), followed by HuPXR/CAR KO (1.7-fold) and mPXR/HuCAR (1.6-fold). Finally, variations are also seen in POR response to letrozole therapy. No induction is seen in in the wild-type, PXR KO, HuPXR and mPXR models in males. In females, significant induction is observed in the PXR/CAR DHu model only.

4.3.1.2 ANASTROZOLE

PXR/CAR status is also a key determinant of anastrozole-induced upregulation in both males **(Figure 4.3.3)** and females **(Figure 4.3.4)**. Once again, no inductions are seen in Cyp1a expression. There is no significant induction of Cyp2a in males. Perturbations in Cyp2c expression were not seen in any of the tested models in either sex, with two exceptions: induction is seen in 1) the mPXR/CAR KO model in males (1.7-fold), and 2) the PXR/CAR DHu model in females (1.9-fold). No induction of POR is observed in either sex after anastrozole treatment, with the exception of the PXR/CAR DHu model in females (1.7-fold).

As with letrozole analysis, the most interesting results are seen in Cyp2b10 and Cyp3a11 expression. As observed following letrozole treatment, expression of Cyp2b10 is absent in any of the CAR KO models, highlighting the crucial role of CAR in the regulation of this enzyme. In terms of induction pattern, anastrozole

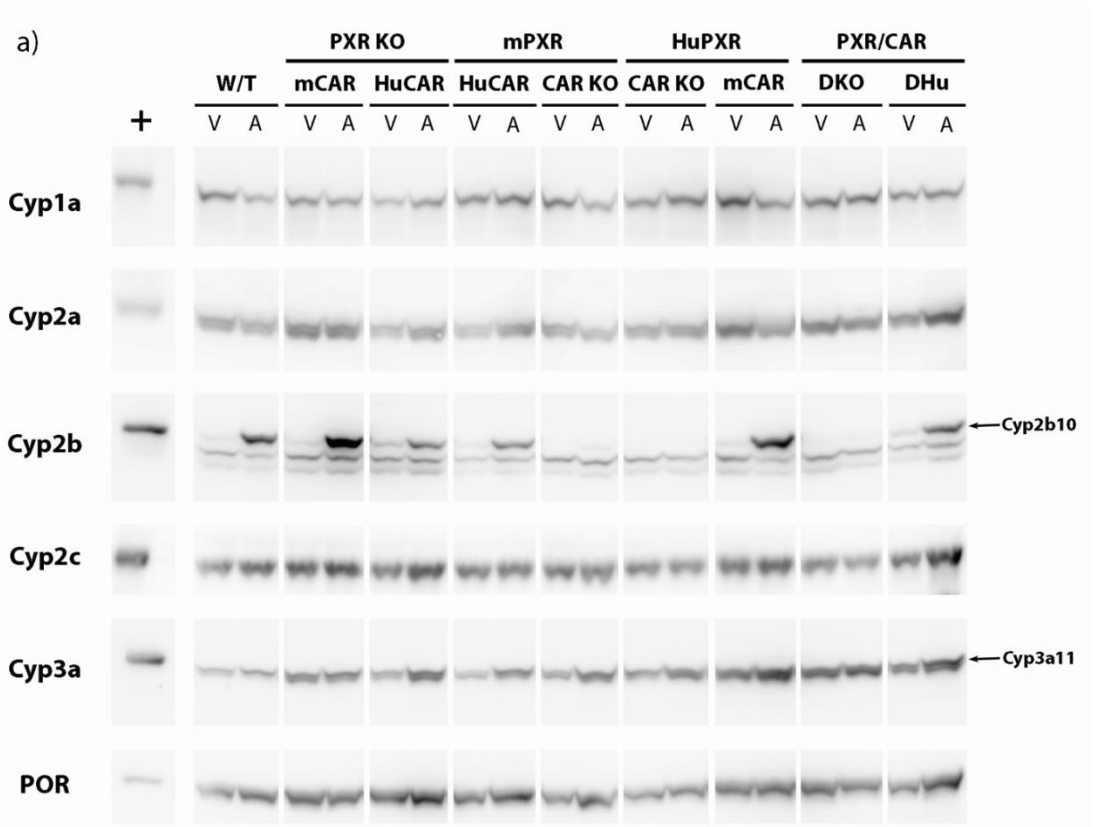


b)

Gene	Genotype								
	W/T	PXR KO		mPXR		HuPXR		PXR/CAR	
		mCAR	HuCAR	HuCAR	CAR KO	CAR KO	mCAR	DKO	DHu
Cyp1a	1.1	0.9	1.1	0.7	1.1	1.2	1.3	0.9	0.7
Cyp2a	1.1	0.8	0.8	0.8	1.5	0.9	0.9	0.6	0.7
Cyp2b	1.7	2.5	1.6	1.8	1.0	0.9	8.6	0.7	2.0
Cyp2c	0.7	1.2	1.3	0.8	1.7	0.8	0.9	1.0	1.4
Cyp3a	1.2	1.0	0.7	3.3	2.0	2.7	0.8	0.7	1.4
POR	0.6	0.6	1.3	1.0	1.4	1.1	1.3	0.9	0.8

Figure 4.3.3: Induction of Cytochrome P450s in male wild type C57BL/6J and transgenic mouse models following 3 day treatment with 20 mg/kg Anastrozole

C57BL/6J mice and a panel of PXR and CAR transgenic models (n=3 per group) were treated p.o. daily with either 20 mg/kg Anastrozole (A) or 0.9% Saline vehicle control (V) for 3 days. a) Western blots of pooled liver microsomes (n = 3 per group; 20μg/well). + = positive control - Liver microsomes from male C57BL/6J mouse treated with either phenobarbital (80 mg/kg/day for 3 days; 7.5μg/well; Cyp1a, 2a, 2b, and 2c) or dexamethasone (50 mg/kg/day for 3 days; 5μg/well; Cyp3a and POR). b) Densitometry analysis - Fold induction/repression following drug treatment relative to saline control. Numbers in red indicate >2 fold induction. Densitometry is isoform specific for Cyp2b10 and Cyp3a11 only. All other data represent densitometry for the full gene cluster.



b)

Gene	Genotype								
	W/T	PXR KO		mPXR		HuPXR		PXR/CAR	
		mCAR	HuCAR	HuCAR	CAR KO	CAR KO	mCAR	DKO	DHu
Cyp1a	0.6	0.9	1.4	1.3	0.7	1.4	0.7	0.9	1.1
Cyp2a	0.9	1.0	1.4	1.6	0.8	1.2	0.8	0.9	1.6
Cyp2b	4.6	6.1	1.6	2.9	1.1	1.0	5.1	0.6	3.7
Cyp2c	1.5	1.2	1.4	1.0	1.1	0.9	1.2	0.8	1.9
Cyp3a	1.3	1.0	1.9	1.8	1.6	1.4	1.4	1.0	1.6
POR	1.6	0.9	1.3	1.3	1.4	1.2	1.1	0.8	1.7

Figure 4.3.4: Induction of Cytochrome P450s in female wild type C57BL/6J and transgenic mouse models following 3 day treatment with 20 mg/kg Anastrozole

C57BL/6J mice and a panel of PXR and CAR transgenic models (n=3 per group) were treated p.o. daily with either 20 mg/kg Anastrozole (A) or 0.9% Saline vehicle control (V) for 3 days. a) Western blots of pooled liver microsomes (n = 3 per group; 20μg/well). + = positive control - Liver microsomes from male C57BL/6J mouse treated with either phenobarbital (80 mg/kg/day for 3 days; 7.5μg/well; Cyp1a, 2a, 2b, and 2c) or dexamethasone (50 mg/kg/day for 3 days; 5μg/well; Cyp3a and POR). b) Densitometry analysis - Fold induction/repression following drug treatment relative to saline control. Numbers in red indicate >2 fold induction. Densitometry is isoform specific for Cyp2b10 and Cyp3a11 only. All other data represent densitometry for the full gene cluster.

induces expression in all genotypes possessing a CAR moiety in both males and females, although the magnitude of expression varies between sexes. In males, the strongest induction is seen in the HuPXR/mCAR model (8.6-fold) followed by the PXR KO/mCAR (2.5-fold) and PXR/CAR DHu (2-fold) models. Induction in wild-type and HuCAR models, whilst notable, does not exceed a two-fold induction threshold. In females, the induction profile is slightly altered, with the strongest induction being recorded in the PXR KO/mCAR model (6.1-fold), closely followed by the HuPXR/mCAR (5.1-fold) and wild-type (4.6-fold). The strongest induction in models containing hCAR is seen in the PXR/CAR DHu model (3.7-fold), followed by the mPXR/HuCAR (2.9-fold) and PXR KO/HuCAR (1.6-fold) models.

The strongest inductions in Cyp3a11 expression following anastrozole treatment are seen in male mice, with the mPXR/HuCAR model demonstrating the highest induction (3.3-fold), followed by the HuPXR/CAR KO (2.7-fold) and mPXR/CAR KO (2-fold) models. Induction was not seen in other models. In females, induction is seen in the HuCAR models, mPXR/CAR KO model and PXR/CAR DHu model, although none of these exceed 2-fold. Induction was not observed in other models.

4.3.2 PROTEIN INDUCTION IS ASSOCIATED WITH INCREASED ENZYME ACTIVITY

Understanding whether protein induction is associated with a change in enzyme activity is vital to our understanding of the pharmacokinetics of these drugs, and therefore coumarin and resorufin assays were analysed with respect to the Western blots discussed in *Section 4.3.1*. All enzyme data is expressed as induction relative to vehicle control samples ± S.E.M, with statistical data describing induction relative to that observed in the wild-type.

4.3.2.1 MALES

Enzyme activity accords well with Western blot data when examining BFC and BQ **(Figure 4.3.5)**, the probe substrates associated with Cyp3a, Cyp1a and Cyp2c activity, particularly when considering letrozole. There is no induction in

BFC or BQ activity in the wild-type model following treatment with either drug, mirroring data from the Western blots. No induction in enzyme activity is seen in the PXR KO, HuPXR or PXR/CAR DHu models. A non-significant repression in activity is observed in the PXR/CAR DKO model. Significant enzyme induction is seen for both BFC and BQ assays in the mPXR/CAR KO (4.0- vs 2.9-fold respectively) and mPXR/HuCAR (4.7- vs 3.5-fold respectively) models, representative of increased Cyp3a activity following letrozole treatment. These findings accord well with protein induction data with the exception of the HuPXR/CAR KO model, in which induction in Cyp3a protein does not translate to an increase in enzyme activity. Data following anastrozole treatment shows a similar pattern, with significant increases in enzyme activity relative to wild-type being recorded in the mPXR/CAR KO and mPXR/HuCAR models, indicating that protein induction in these models does translate to an increase in enzyme activity. Although a modest, non-significant induction in enzyme activity relative to wild-type is observed in the HuPXR/CAR KO model, it does not equate to the magnitude of induction observed on Western blot. Also, non-significant induction in BFC activity appears in the PXR KO/mCAR and HuPXR/mCAR models, which was not predicted by Western blot analysis.

When analysing EFC and MFC **(Figure 4.3.5)**, the probes associated with Cyp2b, Cyp2c and Cyp2e activity, a similar scenario is evident. Unlike in the BFC and BQ assays, there is an induction of approximately 2-fold in both EFC and MFC activity following letrozole treatment in wild-type mice. Induction at wild-type levels is seen in the PXR KO/mCAR, HuPXR/CAR KO, HuPXR/mCAR and PXR/CAR DHu models. Activity is only significantly increased relative to wild-type in the mPXR/HuCAR model in the EFC assay, although not in MFC. There is also a significant repression in EFC activity relative to wild-type following letrozole treatment in the PXR/CAR DKO and PXR KO/HuCAR models, with no induction relative to vehicle control being seen. This is also reflected in the MFC activity assay, although the repression is non-significant. Following anastrozole treatment, induction of approximately 3-fold is observed in both assays in the wild-type model. Similar levels of activity are seen in both assays for the mPXR/HuCAR model, with lower induction of approximately 2-fold in the PXR

Substrate (S)	Enzyme detected
7-benzyloxy-4-trifluoromethyl-coumarin (BFC)	CYP3A4 CYP1A2 CYP2C19 CYP1B1 CYP1A1
7-ethoxy-4-trifluoromethyl-coumarin (EFC)	CYP2B6 CYP2C19 CYP2E1
7-methoxy-4-trifluoromethyl-coumarin (MFC)	CYP2C9 CYP2B6 CYP2E1
7-benzyloxy-quinoline (BQ)	CYP3A4 CYP1A1/2

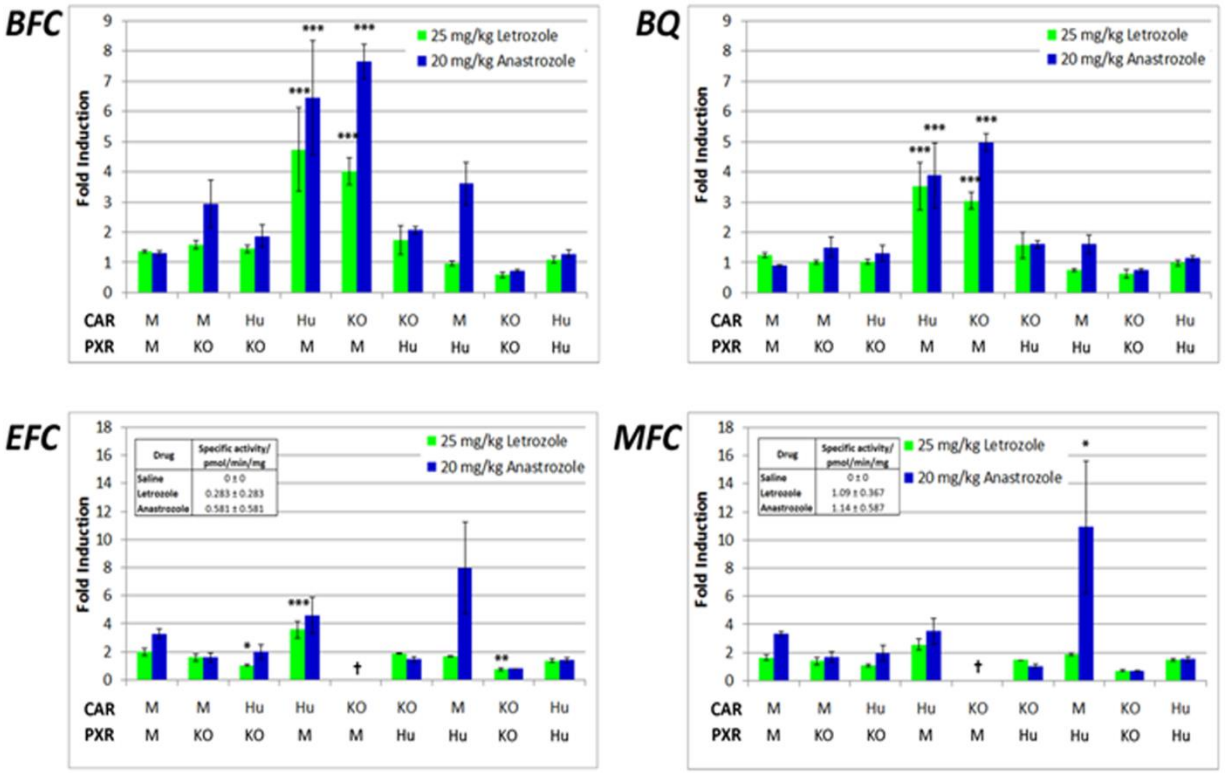
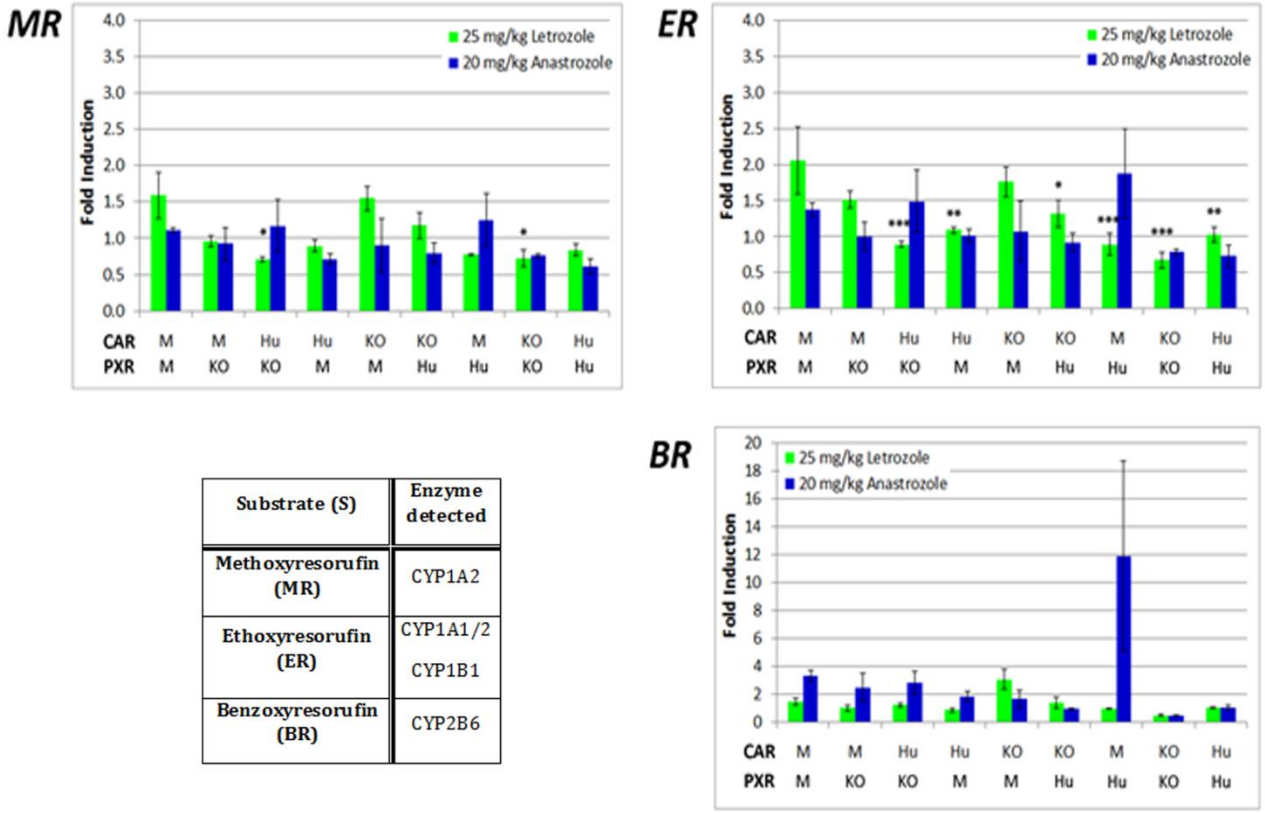


Figure 4.3.5: Cytochrome P450 activity in a panel of male transgenic PXR and CAR mouse models treated with 25 mg/kg Letrozole or 20 mg/kg Anastrozole, as measured by fluorescent coumarin assay

*C57BL/6J mice and a panel of PXR and CAR transgenic mice (n=3 per group; male) were treated p.o. daily with either 0.9% Saline, Letrozole (25 mg/kg/day) or Anastrozole (20 mg/kg/day) for 3 days. Cytochrome P450 activity in liver microsomes was assessed using a panel of coumarin probes (human P450 specificity in table). Data is expressed as fold induction/repression relative to vehicle control ± S.E.M. X-axis indicates genotype: m = murine, KO = knock-out, Hu = Human. Statistics analysed average induction/repression relative to vehicle control for each genotype relative to W/T model. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001. † = Saline control activity = 0 therefore analysis could not be performed. See inset table for specific activity data ± S.E.M.*



Substrate (S)	Enzyme detected
Methoxyresorufin (MR)	CYP1A2
Ethoxyresorufin (ER)	CYP1A1/2 CYP1B1
Benzoxyresorufin (BR)	CYP2B6

Figure 4.3.6: Cytochrome P450 activity in a panel of male transgenic PXR and CAR mouse models treated with 25 mg/kg Letrozole or 20 mg/kg Anastrozole, as measured by fluorescent resorufin assay

*C57BL/6J mice and a panel of PXR and CAR transgenic mice (n=3 per group; male) were treated p.o. daily with either 0.9% Saline, Letrozole (25 mg/kg/day) or Anastrozole (20 mg/kg/day) for 3 days. Cytochrome P450 activity in liver microsomes was assessed using a panel of resorufin probes (human P450 specificity in table). Data is expressed as fold induction/repression relative to vehicle control ± S.E.M. X-axis indicates genotype: m = murine, KO = knock-out, Hu = Human. Statistics analysed average induction/repression relative to vehicle control for each genotype relative to W/T model. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.*

KO, HuPXR/CAR KO and PXR/CAR DHu models. None of these changes are significant relative to wild-type. Enzyme activity is induced relative to wild-type in both assays in the HuPXR/mCAR model only, although this is only significant in the MFC assay. This is the model in which the strongest induction of Cyp2b was recorded on Western blot. A non-significant repression in enzyme activity relative to wild-type is seen in the PXR/CAR DKO, with no induction being recorded in this model relative to vehicle control. Finally, EFC and MFC activity is negligible in the mPXR/CAR KO model in both the basal and induced states, regardless of treatment cohort, indicating that this model is effectively incapable of metabolising these probes.

To provide more specific data regarding isoform activation, a panel of resorufin probes was employed **(Figure 4.3.6)**. On Western blot, it was observed that Cyp1a was not induced in any genotype with either letrozole or anastrozole. This is largely corroborated by the results of MR and ER activity assays which are more specific for Cyp1a isoforms than the coumarins, in which no notable induction in expression is observed, with the exception of the wild-type model. There is a significant repression of MR activity observed in the PXR/CAR DKO and PXR KO/HuCAR models relative to wild-type, following letrozole treatment. No significant change in MR activity was recorded in any other model with letrozole, and in none of the models following anastrazole treatment. Lower activity than wild-type is also a feature of the ER activity assay, with significantly reduced turnover of this probe relative to the wild-type model identified in the PXR/CAR DKO, PXR KO/HuCAR, HuPXR/mCAR, HuPXR/CAR KO, mPXR/HuCAR and PXR CAR DHu models after letrozole treatment. For letrozole, the highest level of induction is seen in the wild-type model, in which a 2-fold induction is observed. The BR assay, which has a greater specificity for Cyp2b than the EFC and MFC assays, was also employed. Induction relative to wild-type was only seen in the mPXR/CAR KO model following letrozole treatment, and was strongest in the HuPXR/mCAR model following anastrozole treatment. All inductions were non-significant relative to wild-type and only that identified following anastrozole treatment agrees with Western blot data. No induction was recorded in any other model for either drug.

4.3.2.2 FEMALES

Protein induction data following letrozole treatment in females indicates that Cyp3a11 is only induced in PXR/CAR DHu, HuPXR/CAR KO and mPXR/HuCAR models. Cyp2c is also induced in the mPXR/HuCAR model, a fact that could have implications for BFC activity. Notable increases in BFC activity **(Figure 4.3.7)** are seen in the mPXR/HuCAR and PXR/CAR DHu models, although these are non-significant relative to wild-type. No induction is seen in any of the other models. However, when we examine data from the more specific BQ activity assay following letrozole treatment **(Figure 4.3.7)**, significant induction in cyp3a11 activity is observed in the mPXR/HuCAR model only. Following anastrozole treatment, induction in BFC activity relative to vehicle control is seen in all models with the exception of the PXR/CAR DKO **(Figure 4.3.7)**. There is a significant induction in BFC activity relative to wild-type following anastrozole treatment in the mPXR/HuCAR model, and activity is also notably increased, albeit non-significantly, in the PXR KO/HuCAR and mPXR/CAR KO models. However, there were no significant inductions relative to wild-type observed in the BQ assay following anastrozole treatment, with notable induction being seen in the mPXR/HuCAR and PXR/CAR DHu models; No induction in response to anastrozole treatment is recorded in the wild-type BQ assay **(Figure 4.3.7)**.

Changes in EFC and MFC activity are also observed, with a significant induction relative to wild-type being identified for both probes in the mPXR/HuCAR model in response to letrozole treatment. Activity was also induced in response to letrozole treatment in the PXR/CAR DHu model, being significantly induced relative to wild-type in the MFC, and non-significantly in the EFC assays. No notable induction relative to vehicle control for EFC or MFC is observed in any other model with letrozole treatment. The induction profile is very different following anastrozole treatment. Enzyme activity in the EFC assay was strongly induced in the wild-type (7-fold), mPXR/HuCAR (10-fold), and HuPXR/mCAR (7-fold) models. Induction relative to vehicle control was also recorded in the PXR KO/HuCAR, PXR KO/mCAR and PXR/CAR DHu models (4-5 -fold).

Substrate (S)	Enzyme detected
7-benzyloxy-4-trifluoromethyl-coumarin (BFC)	CYP3A4 CYP1A2 CYP2C19 CYP1B1 CYP1A1
7-ethoxy-4-trifluoromethyl-coumarin (EFC)	CYP2B6 CYP2C19 CYP2E1
7-methoxy-4-trifluoromethyl-coumarin (MFC)	CYP2C9 CYP2B6 CYP2E1
7-benzyloxy-quinoline (BQ)	CYP3A4 CYP1A1/2

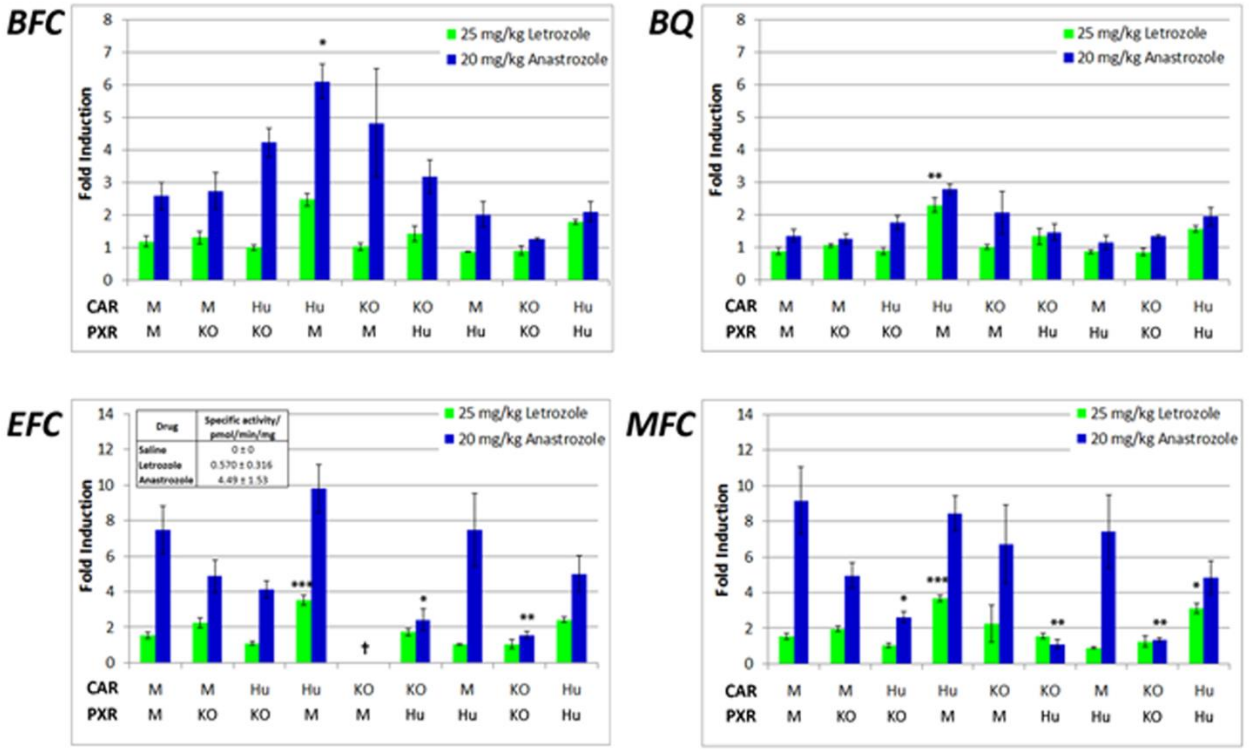
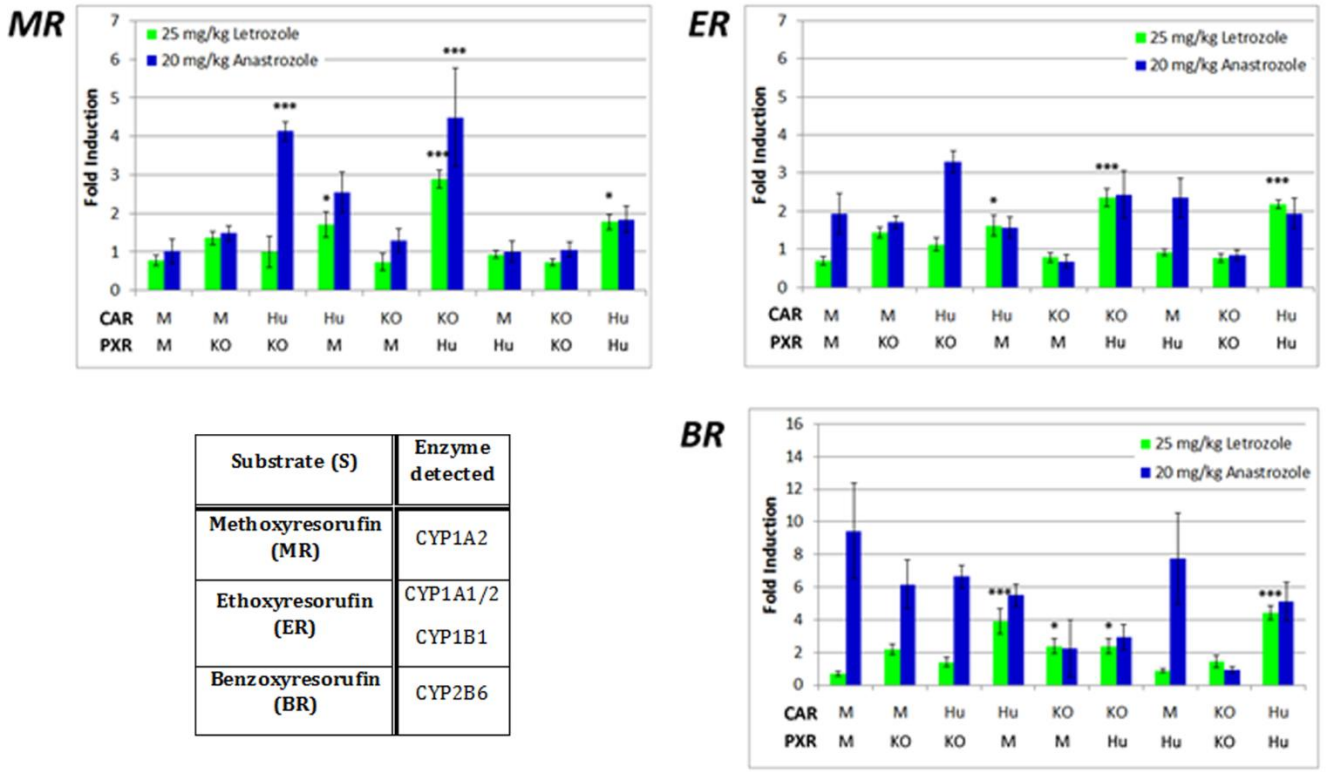


Figure 4.3.7: Cytochrome P450 activity in a panel of female transgenic PXR and CAR mouse models treated with 25 mg/kg Letrozole or 20 mg/kg Anastrozole, as measured by fluorescent coumarin assay

*C57BL/6J mice and a panel of PXR and CAR transgenic mice (n=3 per group; female) were treated p.o. daily with either 0.9% Saline, Letrozole (25 mg/kg/day) or Anastrozole (20 mg/kg/day) for 3 days. Cytochrome P450 activity in liver microsomes was assessed using a panel of coumarin probes (human P450 specificity in table). Data is expressed as fold induction/repression relative to vehicle control ± S.E.M. X-axis indicates genotype: m = murine, KO = knock-out, Hu = Human. Statistics analysed average induction/repression relative to vehicle control for each genotype relative to W/T model. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001. † = Saline control activity = 0 therefore analysis could not be performed. See inset table for specific activity data ± S.E.M.*



Substrate (S)	Enzyme detected
Methoxyresorufin (MR)	CYP1A2
Ethoxyresorufin (ER)	CYP1A1/2 CYP1B1
Benzoxyresorufin (BR)	CYP2B6

Figure 4.3.8: Cytochrome P450 activity in a panel of female transgenic PXR and CAR mouse models treated with 25 mg/kg Letrozole or 20 mg/kg Anastrozole, as measured by fluorescent resorufin assay

*C57BL/6J mice and a panel of PXR and CAR transgenic mice (n=3 per group; female) were treated p.o. daily with either 0.9% Saline, Letrozole (25 mg/kg/day) or Anastrozole (20 mg/kg/day) for 3 days. Cytochrome P450 activity in liver microsomes was assessed using a panel of resorufin probes (human P450 specificity in table). Data is expressed as fold induction/repression relative to vehicle control ± S.E.M. X-axis indicates genotype: m = murine, KO = knock-out, Hu = Human. Statistics analysed average induction/repression relative to vehicle control for each genotype relative to W/T model. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.*

However, these inductions were non-significant relative to wild-type. Activity was significantly reduced relative to wild-type in the HuPXR/CAR KO and PXR/CAR DKO models, with induction being weak in the former and absent in the latter. Once again, no notable activity, either basal or induced, was recorded for the mPXR/CAR KO model in the EFC assay following both treatments. Although certain trends are repeated in the MFC assay, there are marked differences in comparison to the EFC. Firstly, the strongest induction relative to vehicle control is found in the wild-type model (9-fold), with strong induction also identified in the mPXR/HuCAR, mPXR/CAR KO and HuPXR/mCAR models. Induction is also identified in the PXR KO/mCAR and PXR/CAR DHu models at similar levels to those observed in the EFC assay (5-fold). Once again, none of these changes are significant relative to wild-type. However, low levels of induction are observed in the PXR KO/HuCAR model (2-3 -fold), with no induction in the HuPXR/CAR KO and PXR/CAR DKO models. These three models therefore demonstrate significant repression in activity relative to wild-type.

Interestingly, the resorufin probes MR and ER **(Figure 4.3.8)** identify a significant Cyp1a induction following letrozole treatment in females. Extremely significant induction relative to wild-type was seen in the HuPXR/CAR KO model, with a significant but lower activity identified in the mPXR/HuCAR model in both assays. Significant induction in MR activity relative to wild-type was also identified in the PXR/CAR DHu model, increasing to extremely significant in the ER assay. No induction relative to vehicle control was seen in any of the other letrozole treated models, including wild-type. MR activity following anastrozole treatment is significantly induced relative to wild-type in the PXR KO/HuCAR and HuPXR/CAR KO models, with non-significant, but notable, inductions in the mPXR/HuCAR and PXR/CAR DHu models. Unlike in the MR assay, induction of ER activity relative to vehicle control is seen in the wild-type model, with comparable increases seen in the PXR KO/mCAR, PXR KO/HuCAR, mPXR/HuCAR, mPXR/CAR KO, HuPXR/CAR KO, HuPXR/mCAR and PXR/CAR DHu models. Of these, there is a trend towards higher induction relative to wild-type in the PXR KO/HuCAR model, although this is non-

significant. No induction relative to vehicle control is seen in the PXR/CAR DKO and mPXR/CAR KO models.

When investigating changes in BR probe activity **(Figure 4.3.8)** following letrozole treatment, no induction relative to vehicle control is seen in the wild-type, PXR KO/HuCAR, HuPXR/mCAR or PXR/CAR DKO models. Significant induction relative to wild-type is seen in the mPXR/HuCAR, mPXR/CAR KO, HuPXR/CAR KO and PXR/CAR DHu models. There is also a notable, but non-signficant, induction in the PXR KO/mCAR model in response to letrozole. Once again, treatment with anastrozole leads to higher induction of enzyme activity relative to vehicle control. The highest activity is observed in the wild-type model (9-fold) with strong inductions also seen in the PXR KO, mPXR/HuCAR, HuPXR/mCAR and PXR/CAR DHu models (5-7 -fold). Weaker inductions were recorded in the CAR KO models (approximately 2-fold) and no induction in the PXR/CAR DKO. None of these changes are considered to be significantly different in comparison to the wild-type model.

4.3.3 LETROZOLE PHARMACOKINETICS DEMONSTRATE TEMPORAL VARIATION AND SEXUAL DIMORPHISM

4.3.3.1 MALES

In addition to understanding the role of PXR and CAR in the control of letrozole pharmacokinetics, our experimental design allows the analysis of temporal variations and sexual dimorphism in response. The pharmacokinetic curves following 1 day and 3 day treatment with letrozole in males are shown in **Figures 4.3.9** and **4.3.10** respectively. **Figure 4.3.9** indicates that, with the exception of the mPXR/HuCAR model, variations in metabolism between any of the models tested in the one day treatment population appear to be minor. Looking at the pharmacokinetic parameters **(Table 4.3.1 a)**, few significant differences are observed, partly as a result of the intra-population variation.

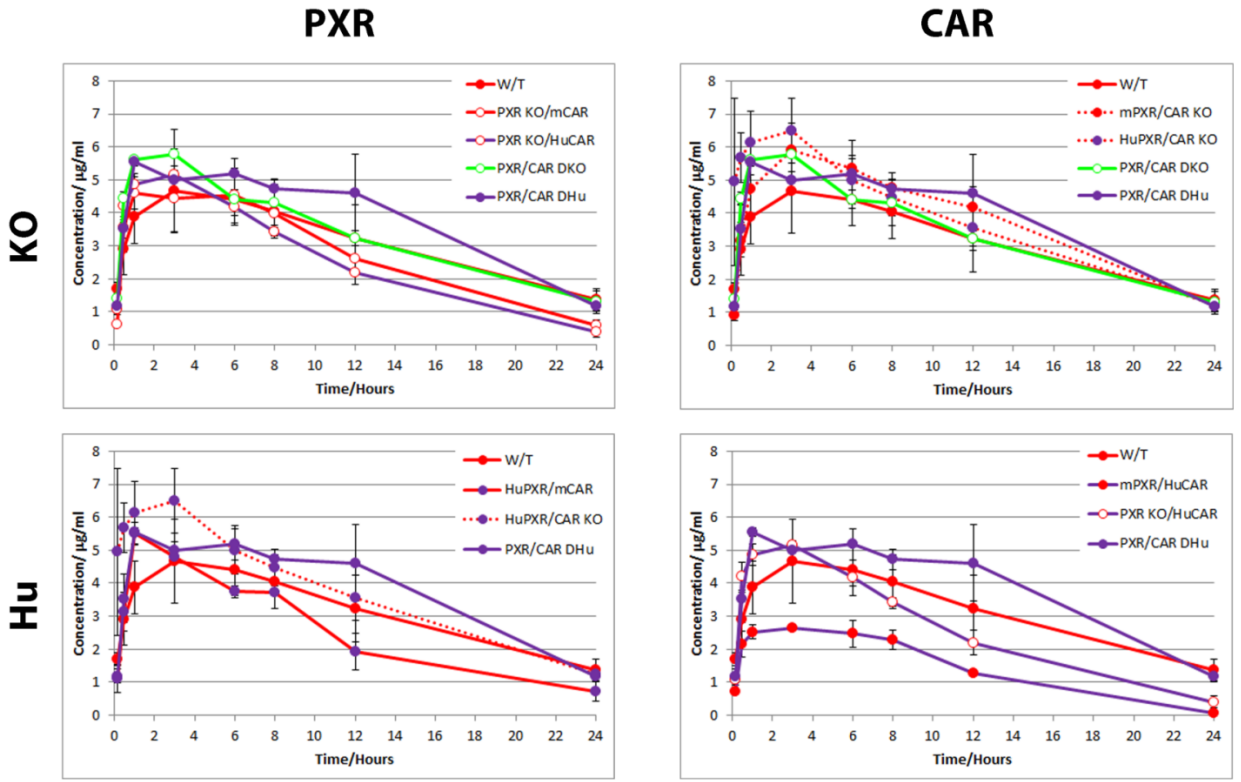


Figure 4.3.9: Pharmacokinetic curves from a range of male PXR and CAR transgenic mouse models following 1 day treatment with 25 mg/kg Letrozole

C57BL/6J mice (W/T) and a panel of PXR and CAR transgenic models (n=3 per group; male) were treated once p.o. with 25 mg/kg Letrozole. Serial blood sampling was performed immediately following drug dose. Drug was extracted using a solvent extraction method. Letrozole concentration was measured by HPLC. Marker indicates PXR genotype (solid red = mPXR, hollow red = PXR KO, solid purple = HuPXR). Line indicates CAR genotype (solid red = mCAR, dashed red = CAR KO, solid purple = HuCAR). Green line indicates PXR/CAR DKO. KO = knockout genotype, Hu = humanized genotype. Error bars indicate S.E.M.

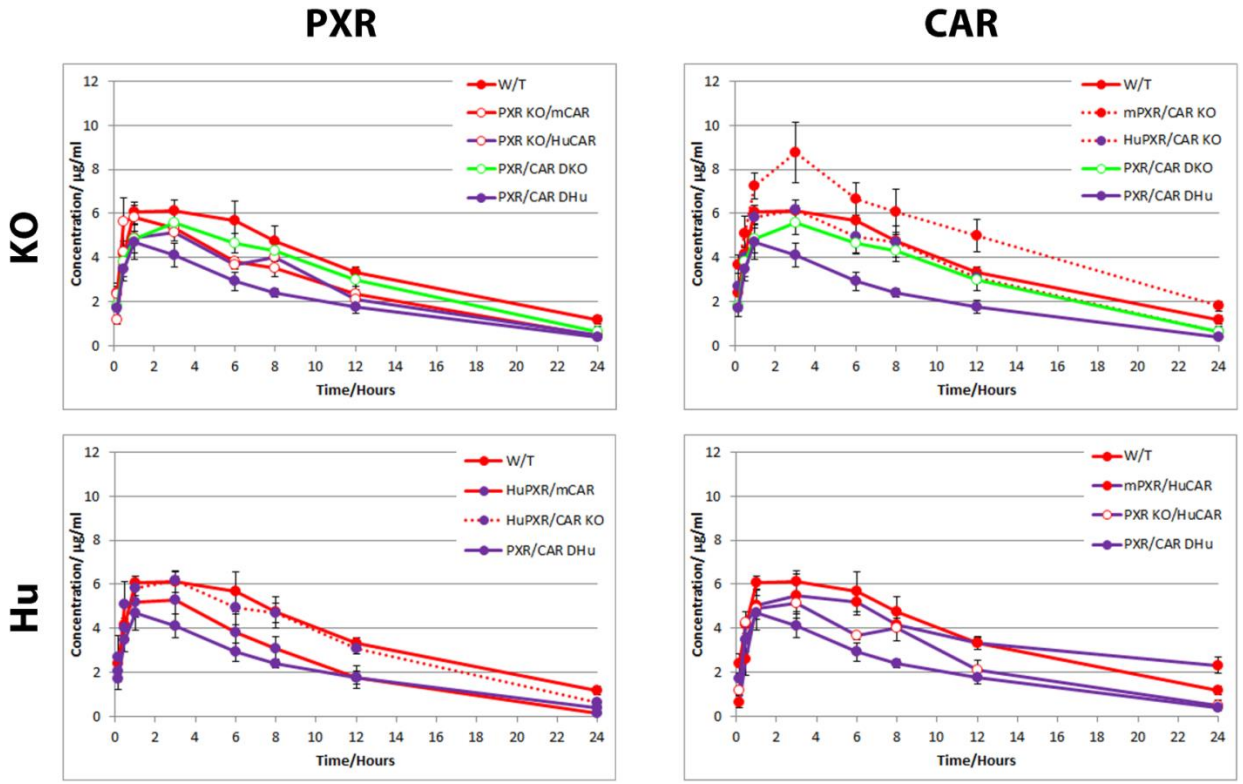


Figure 4.3.10: Pharmacokinetic curves from a range of male PXR and CAR transgenic mouse models following 3 day treatment with 25 mg/kg Letrozole

C57BL/6J mice (W/T) and a panel of PXR and CAR transgenic models (n=3 per group; male) were treated p.o. daily for 3 days with 25 mg/kg Letrozole. Serial blood sampling was performed immediately following final drug dose. Drug was extracted using a solvent extraction method. Letrozole concentration was measured by HPLC. Marker indicates PXR genotype (solid red = mPXR, hollow red = PXR KO, solid purple = HuPXR). Line indicates CAR genotype (solid red = mCAR, dashed red = CAR KO, solid purple = HuCAR). Green line indicates PXR/CAR DKO. KO = knockout genotype, Hu = humanized genotype. Error bars indicate S.E.M.

a)

Genotype	$T_{1/2}$ mins	Cmax µg/ml	Clearance ml/min/kg	AUC (0→1440) min*µg/ml	AUC (0→∞) min*µg/ml
W/T	640 ± 90	4.9 ± 1.0	4.6 ± 0.9	4590 ± 920	5880 ± 1120
PXR KO/mCAR	340 ± 30	5.1 ± 0.8	6.2 ± 1.1	3980 ± 650	4280 ± 760
PXR KO/HuCAR	300 ± 60 *	5.3 ± 0.1	5.6 ± 0.6	3690 ± 280	3890 ± 410
mPXR/HuCAR	280 ± 100 *	3.0 ± 0.2	11.3 ± 0.3 ***	1940 ± 300 *	2220 ± 60 *
mPXR/CAR KO	470 ± 40	5.9 ± 0.8	4.2 ± 0.6	5300 ± 610	6120 ± 730
HuPXR/CAR KO	530 ± 50	8.5 ± 0.8 **	4.0 ± 0.4	5400 ± 560	6350 ± 510
HuPXR/mCAR	420 ± 120	5.5 ± 0.3	6.0 ± 1.0	3890 ± 390	4410 ± 640
PXR/CAR DKO	580 ± 80	6.2 ± 0.4	4.5 ± 0.8	4750 ± 560	5920 ± 990
PXR/CAR DHu	500 ± 50	5.7 ± 0.2	4.3 ± 0.5	5060 ± 440	5940 ± 590

b)

Genotype	$T_{1/2}$ mins	Cmax µg/ml	Clearance ml/min/kg	AUC (0→1440) min*µg/ml	AUC (0→∞) min*µg/ml
W/T	480 ± 20	6.8 ± 0.3	4.2 ± 0.5	5220 ± 460	6040 ± 610
PXR KO/mCAR	320 ± 30	6.1 ± 0.8	6.2 ± 0.6	3910 ± 350	4120 ± 410
PXR KO/HuCAR	350 ± 90	5.4 ± 0.2	6.6 ± 0.9	3600 ± 330	3910 ± 530
mPXR/HuCAR	1070 ± 130 ***	5.8 ± 0.8	3.0 ± 0.5	4210 ± 1160	8800 ± 1350
mPXR/CAR KO	520 ± 30	8.9 ± 1.3	3.0 ± 0.4	7260 ± 950	8620 ± 1080
HuPXR/CAR KO	330 ± 30	6.5 ± 0.2	5.0 ± 0.3	4800 ± 320	5110 ± 320
HuPXR/mCAR	200 ± 20 *	6.0 ± 1.1	8.2 ± 2.0 *	3390 ± 750	3420 ± 760
PXR/CAR DKO	340 ± 40	5.6 ± 0.5	5.3 ± 0.7	4530 ± 530	4880 ± 690
PXR/CAR DHu	380 ± 10	4.7 ± 0.8	8.2 ± 1.0 *	2910 ± 330	3130 ± 360

Table 4.3.1: Pharmacokinetic parameters from a range of male PXR and CAR transgenic mouse models following 1 day or 3 day treatment with 25 mg/kg Letrozole

*C57BL/6J mice (W/T) and a panel of PXR and CAR transgenic models (n=3 per group; male) were treated p.o. daily for 1 (a) or 3 (b) days with 25 mg/kg Letrozole. Serial blood sampling was performed immediately following final drug dose. Drug was extracted using a solvent extraction method. Concentration was measured by HPLC and pharmacokinetic parameters calculated using a non-compartmental method. Data expressed ± S.E.M. Clearance data represents clearance ÷ bioavailability. Statistics analysed each parameter for each genotype relative to W/T model. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.*

The most significant changes relative to wild-type are identified in the mPXR/HuCAR model, with a significantly lower terminal half-life ($t_{½}$) and drug exposure (AUC (0→1440) & AUC (0→∞)), together with an extremely significant increase in clearance. $T_{½}$ is also significantly increased in the PXR KO/mCAR model. Finally, C_{max} is increased in the HuPXR/CAR KO model, although other parameters show no variation.

The first feature evident when examining the curves from the 3 day population **(Figure 4.3.10)** is that there is no drug accumulation in the wild-type model relative to one day treatment, whereas drug exposure in the PXR/CAR DHu model appears to have decreased over the same time period. The latter observation is corroborated by the pharmacokinetic parameters **(Table 4.3.1 b)**, with $t_{½}$ and drug exposure (AUC (0→1440) & AUC (0→∞)) being decreased in the PXR/CAR DHu model, whilst clearance increases indicating faster drug metabolism after 3 days. The next feature is that metabolism in the mPXR/HuCAR model has slowed between 1 and 3 days, with $t_{½}$ approximately quadrupling relative to one day treatment course (1070 ± 133 mins vs 284 ± 98.2 mins). It is approximately twice that recorded in the wild-type model by 3 days (1070 ± 133 mins vs 479 ± 19.2 mins), indicating slower metabolism in this genotype. Although non-significant, clearance is also marginally repressed and absolute drug exposure (AUC (0→∞)) is increased relative to wild-type. This suggests that metabolism has been repressed with continuing treatment with letrozole, leading to drug accumulation and a curve more closely resembling the wild-type model at the 72 hour time-point. Another significant change is the faster $t_{½}$ and increased clearance seen in the HuPXR/mCAR model. This indicates faster drug metabolism and yields a curve more closely resembling that of the PXR/CAR DHu model. However, these changes are abolished in the HuPXR/CAR KO model, with curve and parameters mirroring those of the wild-type model. The differential role of human and mouse PXR is again highlighted by the non-significant drug accumulation seen in the mPXR/CAR KO model. Humanizing CAR has no effect on metabolism, although it does result in faster metabolism when working in synergy with HuPXR, and appears to marginally repress metabolism when working in concert with mPXR.

All parameters are consistent with the wild-type model in the PXR/CAR DKO model.

4.3.3.2 FEMALES

In females, variations in metabolism are more marked than in males. At one day **(Figure 4.3.11)**, it is clear that metabolism is significantly faster and overall drug exposure significantly reduced in the PXR/CAR DHu model relative to wild-type. This is reflected in the PK parameters **(Table 4.3.2 a)**, with C_{max} and drug exposure (AUC (0→1440) & AUC (0→∞)) being significantly reduced and clearance significantly increased. Metabolism is also changed in HuPXR single-transgenic models, with clearance being increased in both, although this is only significant in the HuPXR/CAR KO model. However, in both models absolute drug exposure (AUC (0→∞)) is significantly reduced. Significantly reduced drug exposure is also seen in the PXR KO/HuCAR and mPXR/HuCAR models, with an associated extremely significant increase in clearance in the PXR KO/HuCAR model, and a significant decrease in C_{max} being observed in the mPXR/HuCAR model.. The curve from the mPXR/HuCAR model closely overlays the metabolic curve derived from the PXR/CAR DHu model until the 8 hour time point, at which time metabolism appears to slow. This is reflected in the $t_{½}$, which is similar to that of the wild-type model. Although humanizing PXR and CAR does appear associated with changes in metabolic profile, knocking-out the innate receptors does not exert a significant effect. No significant changes are seen in the PXR KO/mCAR or mPXR/CAR KO models. However, there is a significant change in increase in clearance relative to wild-type seen in the PXR/CAR DKO, associated with a non-significant reduction in drug exposure and $t_{½}$, and with the curve resembling that of the PXR KO/mCAR and mPXR/CAR KO models.

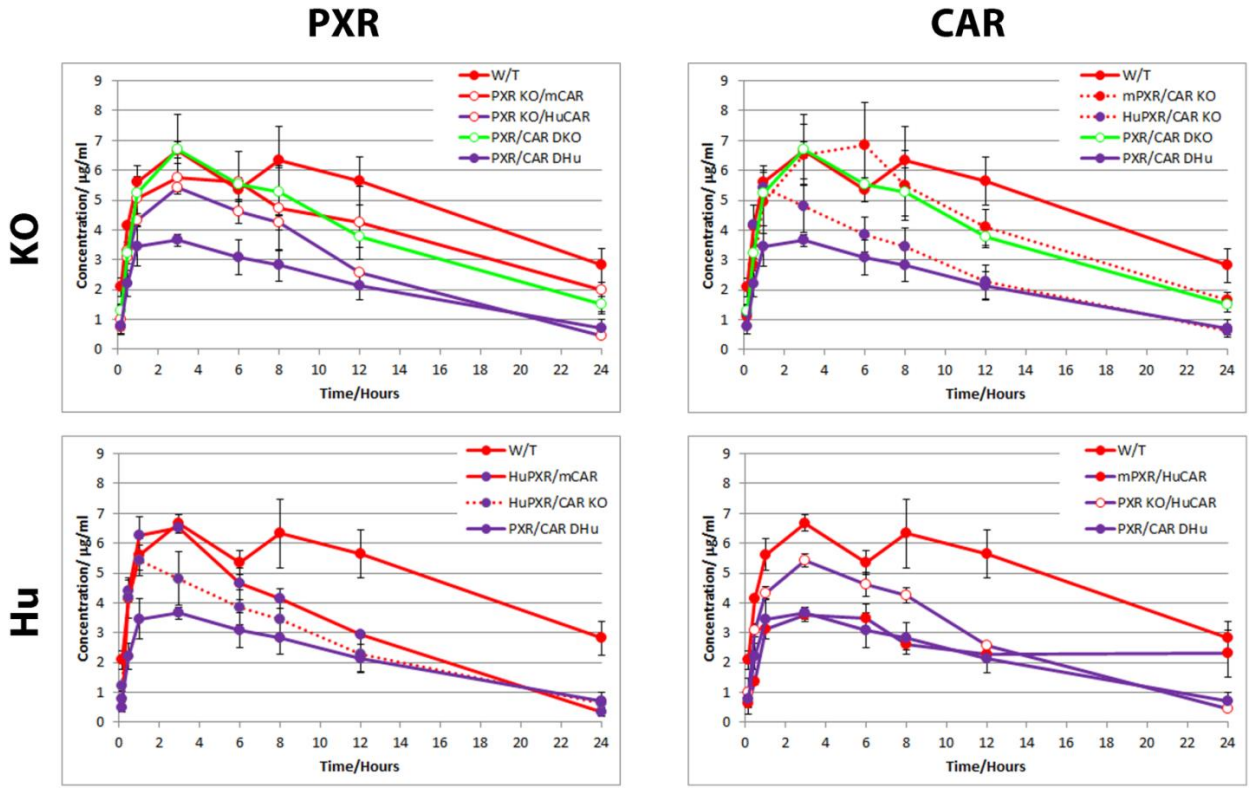


Figure 4.3.11: Pharmacokinetic curves from a range of female PXR and CAR transgenic mouse models following 1 day treatment with 25 mg/kg Letrozole

C57BL/6J mice (W/T) and a panel of PXR and CAR transgenic models (n=3 per group; female) were treated once p.o. with 25 mg/kg Letrozole. Serial blood sampling was performed immediately following drug dose. Drug was extracted using a solvent extraction method. Letrozo le concentration was measured by HPLC. Marker indicates PXR genotype (solid red = mPXR, hollow red = PXR KO, solid purple = HuPXR). Line indicates CAR genotype (solid red = mCAR, dashed red = CAR KO, solid purple = HuCAR). Green line indicates PXR/CAR DKO. KO = knockout genotype, Hu = humanized genotype. Error bars indicate S.E.M.

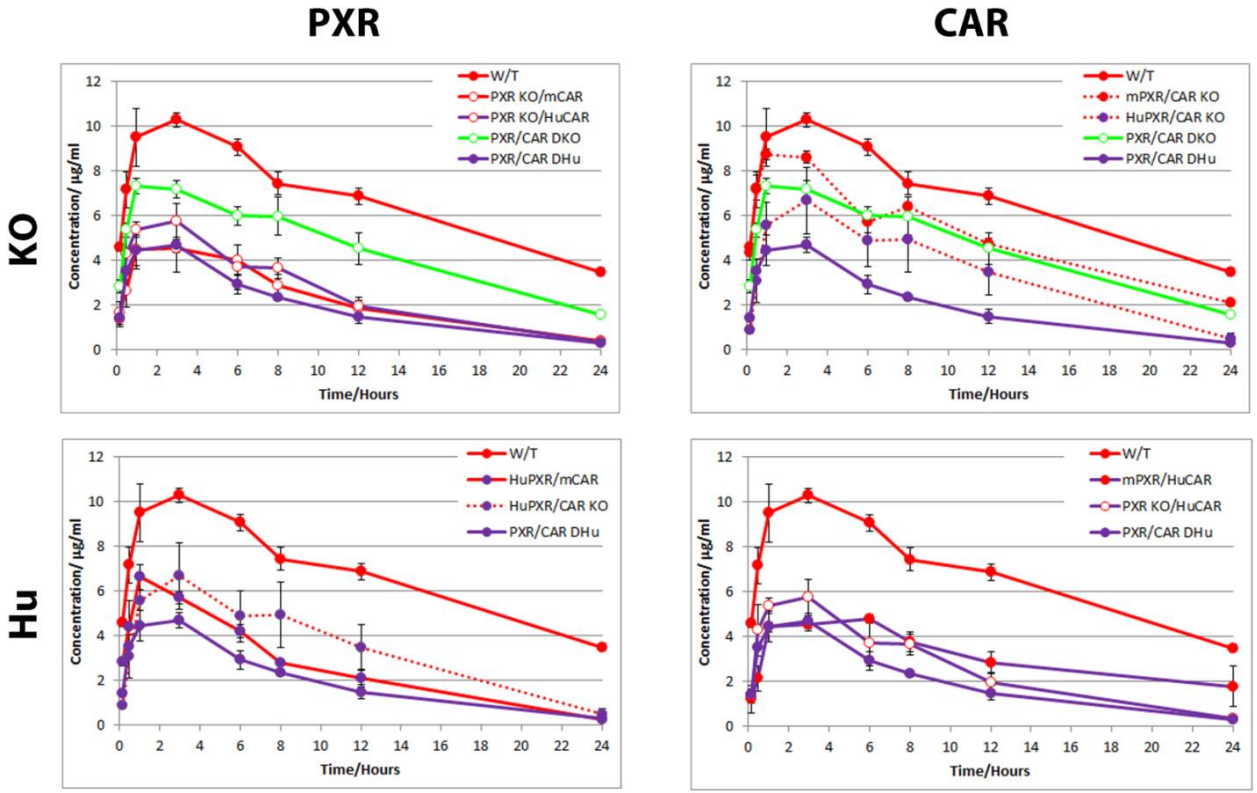


Figure 4.3.12: Pharmacokinetic curves from a range of female PXR and CAR transgenic mouse models following 3 day treatment with 25 mg/kg Letrozole

C57BL/6J mice (W/T) and a panel of PXR and CAR transgenic models (n=3 per group; female) were treated p.o. daily for 3 days with 25 mg/kg Letrozole. Serial blood sampling was performed immediately following final drug dose. Drug was extracted using a solvent extraction method. Letrozole concentration was measured by HPLC. Marker indicates PXR genotype (solid red = mPXR, hollow red = PXR KO, solid purple = HuPXR). Line indicates CAR genotype (solid red = mCAR, dashed red = CAR KO, solid purple = HuCAR). Green line indicates PXR/CAR DKO. KO = knockout genotype, Hu = humanized genotype. Error bars indicate S.E.M.

a)

Genotype	$T_{1/2}$ mins	Cmax µg/ml	Clearance ml/min/kg	AUC (0→1440) min*µg/ml	AUC (0→∞) min*µg/ml
W/T	1030 ± 410	7.2 ± 0.7	2.3 ± 0.5	7090 ± 700	11920 ± 2850
PXR KO/mCAR	750 ± 250	6.2 ± 0.8	3.4 ± 0.9	5840 ± 1240	8380 ± 2040
PXR KO/HuCAR	290 ± 20	5.4 ± 0.2	5.8 ± 0.1 ***	4080 ± 50 *	4270 ± 50 **
mPXR/HuCAR	1130 ± 350	3.9 ± 0.2 *	4.6 ± 0.8	3000 ± 390 **	5850 ± 1200 *
mPXR/CAR KO	570 ± 30	7.0 ± 1.4	3.6 ± 0.7	5970 ± 1060	7320 ± 1220
HuPXR/CAR KO	400 ± 10	5.5 ± 0.6	6.5 ± 1.1 *	3770 ± 710 *	4140 ± 810 **
HuPXR/mCAR	260 ± 40	6.8 ± 0.4	5.4 ± 0.2	4490 ± 110	4630 ± 130 **
PXR/CAR DKO	530 ± 30	7.0 ± 1.0	3.8 ± 0.4 *	5640 ± 570	6830 ± 790
PXR/CAR DHu	460 ± 80	3.9 ± 0.4 *	7.7 ± 1.7 **	3110 ± 610 **	3650 ± 920 **

b)

Genotype	$T_{1/2}$ mins	Cmax µg/ml	Clearance ml/min/kg	AUC (0→1440) min*µg/ml	AUC (0→∞) min*µg/ml
W/T	790 ± 50	10.5 ± 0.6	1.8 ± 0.1	9840 ± 330	13840 ± 660
PXR KO/mCAR	330 ± 20	4.8 ± 0.9 ***	7.7 ± 1.2 ***	3270 ± 590 ***	346 ± 620 ***
PXR KO/HuCAR	280 ± 20	5.6 ± 0.6 ***	7.5 ± 1.2 **	3420 ± 590 ***	3560 ± 640 ***
mPXR/HuCAR	1220 ± 660	4.6 ± 0.2 ***	4.0 ± 1.3	3850 ± 620 ***	8520 ± 3540 *
mPXR/CAR KO	610 ± 20	8.8 ± 0.2	2.8 ± 0.1	7240 ± 260**	9100 ± 440 *
HuPXR/CAR KO	310 ± 10	6.7 ± 1.5 **	5.8 ± 1.4 *	4800 ± 1370***	5040 ± 1470***
HuPXR/mCAR	270 ± 20	6.6 ± 0.6 **	6.7 ± 0.3 **	3650 ± 130 ***	3750 ± 170 ***
PXR/CAR DKO	520 ± 50	7.5 ± 0.4 **	3.3 ± 0.2	6570 ± 510**	7750 ± 440 *
PXR/CAR DHu	330 ± 10	4.9 ± 0.5 ***	8.2 ± 0.7 ***	3000 ± 220 ***	3110 ± 250***

Table 4.3.2: Pharmacokinetic parameters from a range of female PXR and CAR transgenic mouse models following 1 day or 3 day treatment with 25 mg/kg Letrozole

*C57BL/6J mice (W/T) and a panel of PXR and CAR transgenic models (n=3 per group; female) were treated p.o. daily for 1 (a) or 3 (b) days with 25 mg/kg Letrozole. Serial blood sampling was performed immediately following final drug dose. Drug was extracted using a solvent extraction method. Concentration was measured by HPLC and pharmacokinetic parameters calculated using a non-compartmental method. Data expressed ± S.E.M. Clearance data represents clearance ÷ bioavailability. Statistics analysed each parameter for each genotype relative to W/T model. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.*

Variation in the wild-type model reduces the analytical power of the 1 day population, particularly when examining $t_{½}$, in which the variation is so significant that there is insufficient statistical power to provide meaningful analysis. However, although statistical significance cannot be calculated, trends can be seen. Mean $t_{½}$ is strongly reduced in the PXR KO/HuCAR (293 ± 19.8), HuPXR/CAR KO (397 ± 14.4), HuPXR/mCAR (257 ± 44.0) and PXR/CAR DHu (461 ± 76.0) models relative to wild-type (1030 ± 409). It is also reduced in the mPXR/CAR KO and PXR/CAR DKO models, although to a lesser extent. There is no change seen in the mPXR/HuCAR or the PXR KO/mCAR models. It therefore appears that metabolism is faster in many models, particularly in those in which PXR is humanized.

Differential metabolism between the native and humanized models is further accentuated by 3 days **(Figure 4.3.12)**. There is clear drug accumulation, based on AUC data, in the wild-type model between the 1 and 3 day populations, with no change being seen in $t_{½}$ or clearance. However, accumulation is marginal in the PXR/CAR DHu model as a result of the faster metabolism observed in the one day population. When examining the PK parameters **(Table 4.3.2 b)**, significant changes are seen in all parameters in the PXR KO, HuPXR and PXR/CAR DHu models relative to wild-type, with the exception of $t_{½}$ in which statistical power is insufficient to provide valid statistical conclusions. This is corroborated when examining the curves, with those from both PXR KO models closely resembling the PXR/CAR DHu model, and those from the HuPXR models having maintained the profile derived from the 1 day population. Significant reductions in C_{max} and drug exposure are also observed in the PXR/CAR DKO model, indicating faster metabolism than in the wild-type, although only a marginal increase in clearance is recorded. Significant reductions in C_{max} and drug exposure are observed in the HuCAR models, with both mirroring the PXR/CAR DHu profile. However, knocking-out CAR has little effect when coupled with the murine PXR receptor, although faster metabolism is observed when PXR is humanized or knocked-out. No significant changes are seen in C_{max} or clearance in the mPXR/CAR KO model, although there is a significant

reduction in drug exposure, indicating slower metabolism in the wild-type model is primarily driven by native PXR.

4.3.4 ANASTROZOLE PHARMACOKINETICS DEMONSTRATE INTER-SPECIES VARIATION AND SEXUAL DIMORPHISM

4.3.4.1 MALES

From the curves, it is obvious that anastrozole metabolism is different in the PXR/CAR DHu model vs the wild-type following both treatment regimes **(Figure 4.3.13 & 4.3.14)**. At one day **(Figure 4.3.13)**, $t_{½}$ is significantly reduced to approximately 50% that of the wild-type, indicating faster metabolism **(Table 4.3.3 a)**. Although there are no other significant changes in PK parameters with this model, there is a trend towards higher systemic exposure in the PXR/CAR DHu model, with C_{max} and drug exposure (AUC (0→1440) & AUC (0→∞)) being increased, and clearance being decreased. This suggests that drug uptake is more efficient in the PXR/CAR DHu model. Examining the single HuPXR models, the importance of crosstalk between PXR and CAR is highlighted, with increased metabolism being observed in the HuPXR/mCAR model, as indicated by the significant reduction in $t_{½}$ and increase in clearance recorded in this model, together with reduced, although non-significant, drug exposure. However, the reverse is observed in the HuPXR/CAR KO model, with no change in $t_{½}$, a significant increase in C_{max}, increases in drug exposure and reduction in clearance, although the latter two findings are non-significant. Humanizing CAR does not have a significant effect on PK parameters, with marginal trends towards increased drug exposure and C_{max} observed, together with slight reductions in clearance. However, $t_{½}$ is significantly reduced in the PXR KO/HuCAR model, but unchanged in the mPXR/HuCAR model.

Knocking-out receptors does influence metabolism, with the curves derived from both PXR KO models, as well as the PXR/CAR DKO model, shifting towards

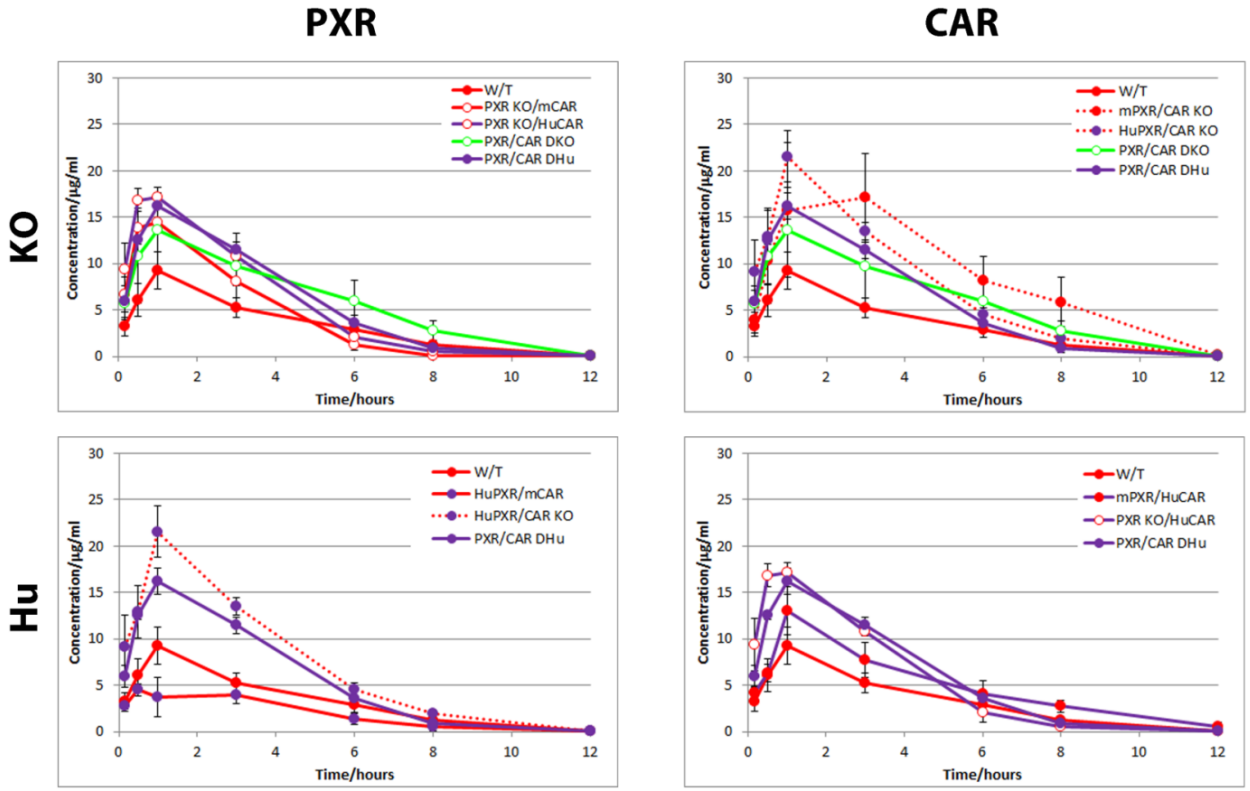


Figure 4.3.13: Pharmacokinetic curves from a range of male PXR and CAR transgenic mouse models following 1 day treatment with 20 mg/kg Anastrozole

C57BL/6J mice (W/T) and a panel of PXR and CAR transgenic models (n=3 per group; male) were treated once p.o. with 20 mg/kg Anastrozole. Serial blood sampling was performed immediately following drug dose. Drug was extracted using a solvent extraction method. Anastrozole concentration was measured by UPLC-MS/MS. Marker indicates PXR genotype (solid red = mPXR, hollow red = PXR KO, solid purple = HuPXR). Line indicates CAR genotype (solid red = mCAR, dashed red = CAR KO, solid purple = HuCAR). Green line indicates PXR/CAR DKO. KO = knockout genotype, Hu = humanized genotype. Error bars indicate S.E.M.

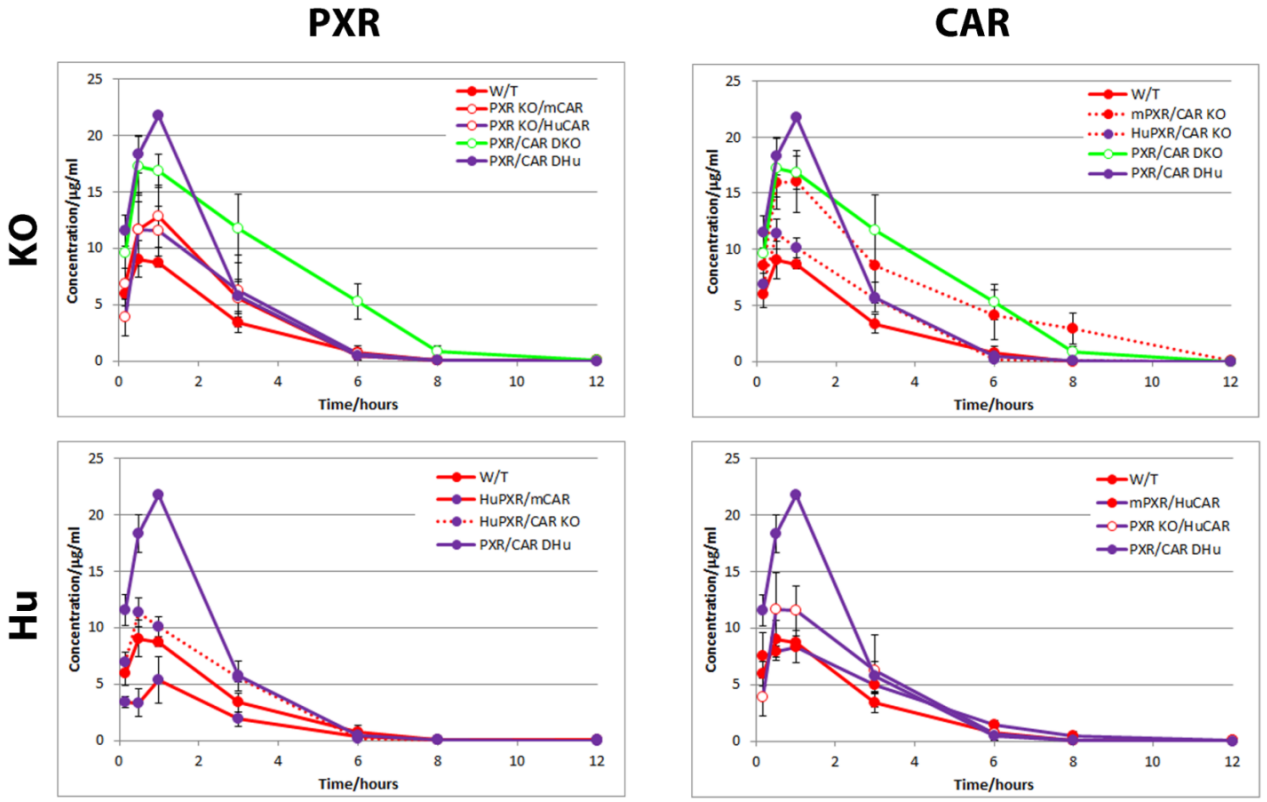


Figure 4.3.14: Pharmacokinetic curves from a range of male PXR and CAR transgenic mouse models following 3 day treatment with 20 mg/kg Anastrozole

C57BL/6J mice (W/T) and a panel of PXR and CAR transgenic models (n=3 per group; male) were treated p.o. daily for 3 days with 20 mg/kg Anastrozole. Serial blood sampling was performed immediately following final drug dose. Drug was extracted using a solvent extraction method. Anastrozole concentration was measured by UPLC-MS/MS. Marker indicates PXR genotype (solid red = mPXR, hollow red = PXR KO, solid purple = HuPXR). Line indicates CAR genotype (solid red = mCAR, dashed red = CAR KO, solid purple = HuCAR). Green line indicates PXR/CAR DKO. KO = knockout genotype, Hu = humanized genotype. Error bars indicate S.E.M.

a)

Genotype	$T_{1/2}$ mins	Cmax µg/ml	Clearance ml/min/kg	AUC (0→1440) min*µg/ml	AUC (0→∞) min*µg/ml
W/T	120 ± 9	9.5 ± 1.8	7.1 ± 0.5	2830 ± 180	2830 ± 183
PXR KO/mCAR	70 ± 17 *	15.1 ± 3.0	6.89 ± 0.5	2940 ± 220	2940 ± 222
PXR KO/HuCAR	50 ± 3 ***	18.1 ± 1.1	4.9 ± 0.2	4090 ± 160	4090 ± 157
mPXR/HuCAR	130 ± 17	13.1 ± 2.6	5.1 ± 0.7	3960 ± 520	4070 ± 630
mPXR/CAR KO	100 ± 5	24.0 ± 4.5 *	3.4 ± 0.8	6460 ± 1260	6460 ± 1260
HuPXR/CAR KO	110 ± 9	21.6 ± 2.7 *	3.9 ± 0.6	5230 ± 870	5440 ± 820
HuPXR/mCAR	50 ± 4 ***	5.8 ± 1.1	15.5 ± 3.8 **	1440 ± 330	1440 ± 330
PXR/CAR DKO	100 ± 2	13.6 ± 4.7	4.8 ± 1.3	5210 ± 1920	5210 ± 1920
PXR/CAR DHu	50 ± 10 **	16.2 ± 1.4	4.9 ± 0.3	4110 ± 250	4130 ± 240

b)

Genotype	$T_{1/2}$ mins†	Cmax µg/ml	Clearance ml/min/kg	AUC (0→1440) min*µg/ml	AUC (0→∞) min*µg/ml
W/T	40 ± 4	9.7 ± 1.2	13.3 ± 0.9	1510 ± 110	1510 ± 110
PXR KO/mCAR	140 ± 80	12.9 ± 2.8	10.5 ± 3.4	2270 ± 550	2270 ± 550
PXR KO/HuCAR	50 ± 4	13.5 ± 2.9	9.5 ± 2.3	2390 ± 640	2400 ± 640
mPXR/HuCAR	90 ± 10	8.7 ± 1.5	10.0 ± 2.1	2180 ± 470	2190 ± 470
mPXR/CAR KO	140 ± 30	17.3 ± 2.1 *	5.68 ± 1.67	4200 ± 1200 *	4200 ± 1200 *
HuPXR/CAR KO	60 ± 10	11.4 ± 1.3	8.7 ± 0.6	2320 ± 190	2330 ± 190
HuPXR/mCAR	50 ± 4	4.1 ± 0.7	29.8 ± 6.7 ***	770 ± 220	770 ± 230
PXR/CAR DKO	100 ± 20	18.6 ± 2.0 *	4.4 ± 0.7	4660 ± 750 **	4760 ± 840 **
PXR/CAR DHu	40 ± 4	21.8 ± 0.2 ***	5.5 ± 0.6	3710 ± 360	3710 ± 360

Table 4.3.3: Pharmacokinetic parameters from a range of male PXR and CAR transgenic mouse models following 1 day or 3 day treatment with 20 mg/kg Anastrozole

*C57BL/6J mice (W/T) and a panel of PXR and CAR transgenic models (n=3 per group; male) were treated p.o. daily for 1 (a) or 3 (b) days with 20 mg/kg Anastrozole. Serial blood sampling was performed immediately following final drug dose. Drug was extracted using a solvent extraction method. Concentration was measured by HPLC and pharmacokinetic parameters calculated using a non-compartmental method. Data expressed ± S.E.M. Clearance data represents clearance ÷ bioavailability. Statistics analysed each parameter for each genotype relative to W/T model. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001. † = statistical power too low for statistical analysis.*

the profile seen in the PXR/CAR DHu. This shift in the PXR KO models is associated with a significant decrease in $t_{½}$, particularly in the PXR KO/HuCAR model. C_{max} is increased in both, with an increase in drug exposure and reduction in clearance being observed in the PXR KO/HuCAR model only. No change in drug exposure or clearance is observed in the PXR KO/mCAR model. None of the latter variations are significant. In the PXR/CAR DKO model, no significant changes are seen as a result of intra-population variation, although there is an indication the C_{max}, clearance and drug exposure have changed in a similar manner to the PXR KO models. Removing CAR appears to have a greater effect on systemic exposure, with significant increases in C_{max} being recorded in the mPXR/CAR KO model, together with near significant increases in drug exposure ($p=0.06$) and a non-significant reduction in clearance, although it's influence on $t_{½}$ is negligible. A similar pattern is seen in the HuPXR/CAR KO model, although intra-population variation is too great to achieve significance.

These differences are more marked in the 3 day population, with metabolic rate appearing to have increased in both wild-type and PXR/CAR DHu models **(Figure 4.3.14)**. Although C_{max} is unchanged in the wild-type model in comparison to the one day population, there is a reduction in $t_{½}$ and drug exposure, and increase in clearance, all suggesting that metabolic rate has increased. In contrast, there is a marked increase in the drug entering the system in the PXR/CAR DHu model, with a significant increase in C_{max} relative to wild-type **(Table 4.3.3 b)**. There are also non-significant increases in drug exposure, and a reduction in clearance in the PXR/CAR DHu model, although $t_{½}$ remains unchanged relative to wild type. The effect of humanizing PXR is dependent on CAR status. In the HuPXR/CAR KO model, the curve resembles that of the wild type. Although there are no significant changes in the PK parameters, there is a trend towards higher systemic exposure, with increased $t_{½}$ and reduced clearance being recorded. There is no change in C_{max} and a marginal increase in drug exposure. However, in the HuPXR/mCAR model there is reduced systemic exposure, with the curve located below that of the wild-type. There is an extremely significant increase in clearance, and this leads to a non-significant reduction in C_{max} and drug exposure. Likewise, the effect of

humanizing CAR is dependent on PXR status, although the variation in effect is not as marked as in the HuPXR models. Again, both curves more closely resemble the wild-type than the PXR/CAR DHu, with changes in PK parameters being non-significant. Indeed, those recorded in the PXR KO/HuCAR model effectively overlay those from the wild-type. However, the mPXR/HuCAR model displays a slower t½ indicative of slower metabolism.

Variation is also pronounced in the knockout models, and particularly in the PXR/CAR DKO where significant increases in C_{max} and drug exposure are observed, together with non-significant increase in t½ and reduction in clearance. This is indicative of slower metabolism and drug accumulation, and reflected in the significantly altered curve. The only single knockout model to reflect this finding is the mPXR/CAR KO model, in which significant increases in C_{max} and drug exposure, together with a non-significant increase in t½ and reduction in clearance. However, marginal increases in drug exposure and t½, and marginal reduction in clearance rate are observed in the HuPXR/CAR KO model. Although both PXR KO curves overlay each other, a notable but non-significant increase in t½ is recorded in the PXR KO/mCAR model. However, this genotype displays significant inter-individual variation in both clearance and t½. In contrast, the PK parameters for the PXR KO/HuCAR model are comparable to those of the wild-type model.

4.3.4.2 FEMALES

Sexual dimorphism is evident in the female response to anastrozole **(Figure 4.3.15 & 4.3.16)**. The first key feature is that drug exposure, clearance and C_{max} are significantly lower in the PXR/CAR DHu model relative to wild-type in the 1 day population **(Table 4.3.4 a)**. This is in contrast to the males, in which significantly higher systemic exposure is seen in the PXR/CAR DHu model relative to wild-type. As in the males, the lowest systemic exposure is seen in the HuPXR/mCAR model, with extremely significant reductions in C_{max} and drug exposure, and increase in clearance. There is also a non-significant reduction in t½. Significant reduction in exposure is also seen in the HuPXR/CAR KO model,

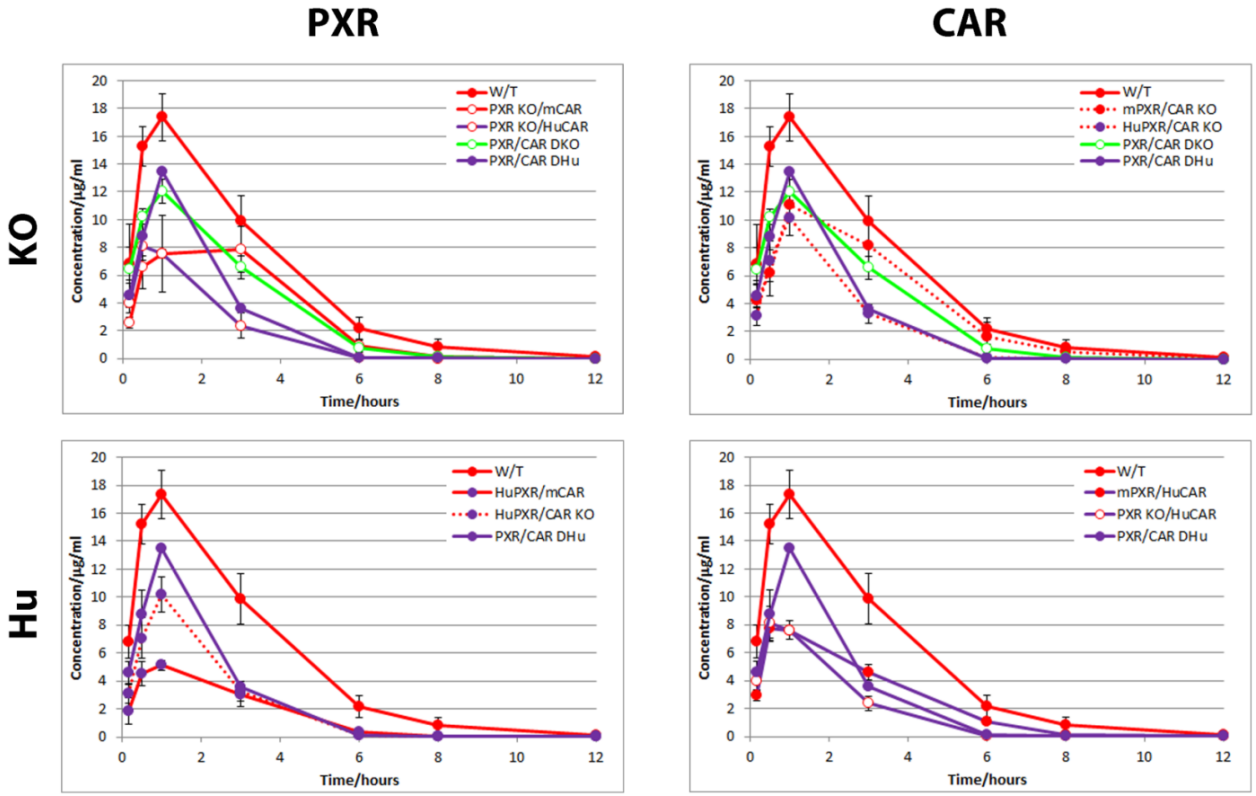


Figure 4.3.15: Pharmacokinetic curves from a range of female PXR and CAR transgenic mouse models following 1 day treatment with 20 mg/kg Anastrozole

C57BL/6J mice (W/T) and a panel of PXR and CAR transgenic models (n=3 per group; female) were treated once p.o. with 20 mg/kg Anastrozole. Serial blood sampling was performed immediately following drug dose. Drug was extracted using a solvent extraction method. Anastrozole concentration was measured by UPLC-MS/MS. Marker indicates PXR genotype (solid red = mPXR, hollow red = PXR KO, solid purple = HuPXR). Line indicates CAR geno type (solid red = mCAR, dashed red = CAR KO, solid purple = HuCAR). Green line indicates PXR/CAR DKO. KO = knockout genotype, Hu = humanized genotype. Error bars indicate S.E.M.

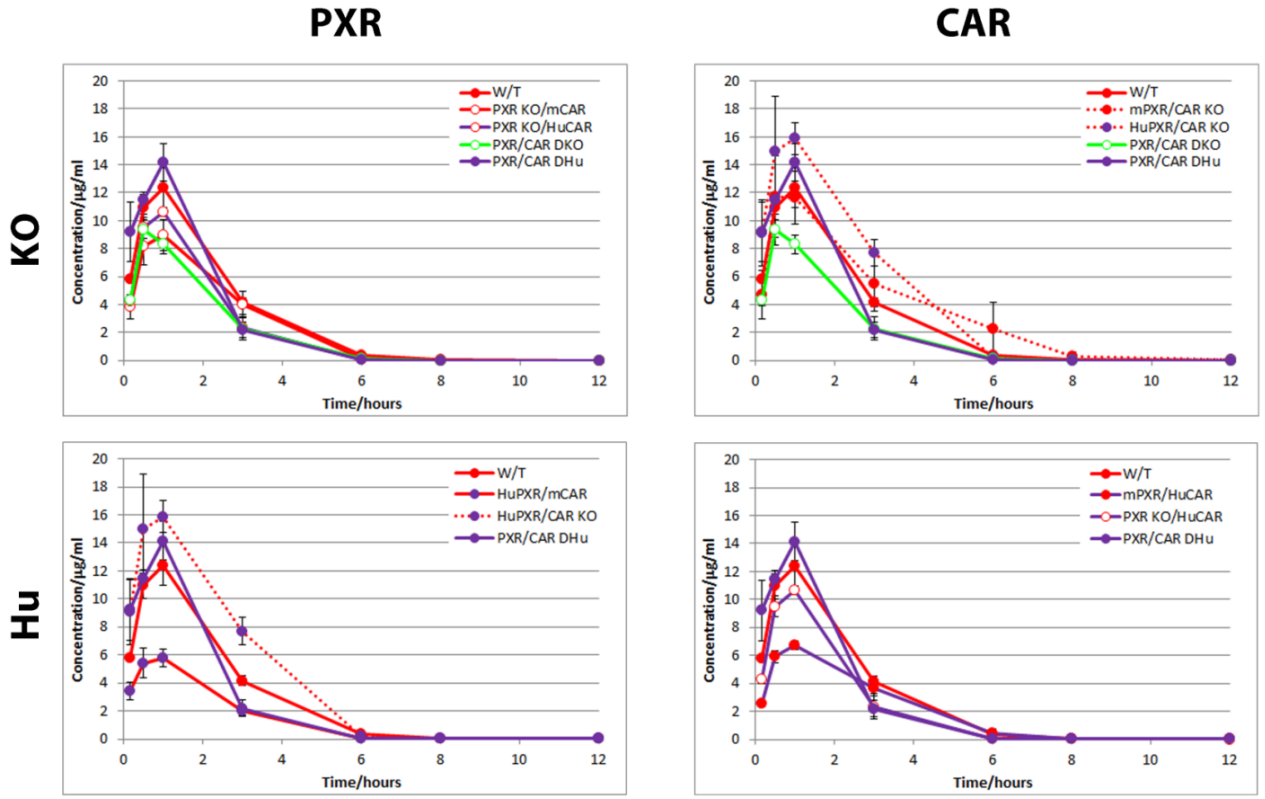


Figure 4.3.16: Pharmacokinetic curves from a range of female PXR and CAR transgenic mouse models following 3 day treatment with 20 mg/kg Anastrozole

C57BL/6J mice (W/T) and a panel of PXR and CAR transgenic models (n=3 per group; female) were treated p.o. daily for 3 days with 20 mg/kg Anastrozole. Serial blood sampling was performed immediately following final drug dose. Drug was extracted using a solvent extraction method. Anastrozole concentration was measured by UPLC-MS/MS. Marker indicates PXR genotype (solid red = mPXR, hollow red = PXR KO, solid purple = HuPXR). Line indicates CAR genotype (solid red = mCAR, dashed red = CAR KO, solid purple = HuCAR). Green line indicates PXR/CAR DKO. KO = knockout genotype, Hu = humanized genotype. Error bars indicate S.E.M.

a)

Genotype	$T_{1/2}$ mins	Cmax µg/ml	Clearance ml/min/kg	AUC (0→1440) min*µg/ml	AUC (0→∞) min*µg/ml
W/T	70 ± 25	17.4 ± 1.7	5.4 ± 1.1	4000 ± 710	4000 ± 710
PXR KO/mCAR	50 ± 2	8.9 ± 1.6***	10.7 ± 2.54	2100 ± 510**	2100 ± 510**
PXR KO/HuCAR	30 ± 2	8.7 ± 0.6***	16.5 ± 1.5***	1230 ± 110***	1230 ± 107***
mPXR/HuCAR	40 ± 4	8.4 ± 0.6***	11.7 ± 0.5**	1710 ± 80**	1710 ± 80**
mPXR/CAR KO	190 ± 35***	11.1 ± 2.3**	8.7 ± 2.5	2660 ± 680*	2670 ± 680*
HuPXR/CAR KO	40 ± 7	10.4 ± 0.9**	13.1 ± 0.1***	1520 ± 10**	1520 ± 10**
HuPXR/mCAR	40 ± 4	5.4 ± 0.3***	20.8 ± 2.1***	980 ± 100***	980 ± 100***
PXR/CAR DKO	60 ± 7	11.6 ± 0.7**	8.6 ± 0.9*	2380 ± 290*	2380 ± 290*
PXR/CAR DHu	70 ± 15	13.0 ± 0.4*	10.1 ± 1.2*	2040 ± 280**	2050 ± 280**

b)

Genotype	$T_{1/2}$ mins	Cmax µg/ml	Clearance ml/min/kg	AUC (0→1440) min*µg/ml	AUC (0→∞) min*µg/ml
W/T	60 ± 10	12.7 ± 1.3	10.0 ± 0.6	2000 ± 110	2000 ± 110
PXR KO/mCAR	150 ± 90	9.0 ± 1.1	13.9 ± 3.0	1560 ± 280	1560 ± 280
PXR KO/HuCAR	40 ± 3	10.8 ± 0.3	11.6 ± 1.4	1770 ± 220	1770 ± 220
mPXR/HuCAR	40 ± 4	6.9 ± 0.2	15.5 ± 1.6*	1320 ± 120	1320 ± 120
mPXR/CAR KO	160 ± 20	12.5 ± 2.5	9.8 ± 2.6	2490 ± 890	2500 ± 880
HuPXR/CAR KO	30 ± 1	17.1 ± 1.8	7.0 ± 0.4	2890 ± 170	2890 ± 170
HuPXR/mCAR	30 ± 1	6.4 ± 0.8*	22.1 ± 2.7***	930 ± 100	930 ± 100
PXR/CAR DKO	50 ± 7	9.8 ± 0.8	16.0 ± 2.1*	1290 ± 160	1290 ± 160
PXR/CAR DHu	50 ± 6	14.7 ± 0.8	9.3 ± 0.8	2010 ± 220	2180 ± 180

Table 4.3.4: Pharmacokinetic parameters from a range of female PXR and CAR transgenic mouse models following 1 day or 3 day treatment with 20 mg/kg Anastrozole

*C57BL/6J mice (W/T) and a panel of PXR and CAR transgenic models (n=3 per group; female) were treated p.o. daily for 1 (a) or 3 (b) days with 20mg/kg Anastrozole. Serial blood sampling was performed immediately following final drug dose. Drug was extracted using a solvent extraction method. Concentration was measured by HPLC and pharmacokinetic parameters calculated using a non-compartmental method. Data expressed ± S.E.M. Clearance data represents clearance ÷ bioavailability. Statistics analysed each parameter for each genotype relative to W/T model. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.*

with C_{max} and drug exposure decreasing, and clearance increasing more than is seen in the PXR/CAR DHu model. There is a non-significant reduction in $t_{½}$ in the HuPXR/CAR KO model relative to wild-type. Similar pharmacokinetic effects are observed in the HuCAR models, with significant changes in all parameters except $t_{½}$. The greatest effect is seen in the PXR KO/HuCAR model, in which clearance approximately triples with respect to wild-type, resulting in significantly lower drug exposure. However, clearance is more than doubled in the mPXR/HuCAR model, and drug exposure more than halved, suggesting that humanizing CAR has a significant effect on anastrozole metabolism in the females. C_{max} does not change between HuCAR models.

Knocking-out both receptors has a significant effect on the metabolism of anastrozole, with C_{max} and drug exposure decreasing and clearance increasing significantly relative to wild-type. The effect on metabolism is very similar to that seen in the PXR/CAR DHu model, again indicating the differing roles of the murine and human receptors. Knocking-out PXR alone has a more significant effect than knocking-out both PXR and CAR, with C_{max} approximately halved relative to wild-type, and clearance and drug exposure significantly altered. The scale of these changes is dependent on CAR status. In the PXR KO/mCAR model, drug exposure is significantly reduced and clearance strongly, although non-significantly, increased. Once again, inter-individual variation is marked in the analysis of drug clearance in this model, accounting for the lack of significance. However, in the PXR KO/HuCAR model, clearance is very significantly increased and drug exposure reduced. $T_{½}$ is non-significantly reduced in both these models. Knocking-out CAR has a much smaller, but significant, effect with both curves resembling those of the PXR/CAR DKO model. However, when looking at the parameters, the importance of PXR status becomes apparent, again highlighting the significant cross-talk between these receptors. Significant reductions in C_{max} and drug exposure are a feature of the mPXR/CAR KO model, and commensurate with the variations observed in the curve. However, a no variation in clearance is seen as a result of inter-individual variability, together with an extremely significant increase in $t_{½}$. However, if we examine the HuPXR/CAR KO model we see greater reductions in

C_{max} and drug exposure, and these are associated with a clearance value more than double that of the wild-type and a non-significant reduction in $t_{½}$. The magnitude of inter-model variation is significantly reduced by 3 days **(Figure 4.3.16)**. Of particular note is the alteration in wild-type pharmacokinetics, in which C_{max} and drug exposure are reduced and clearance increased, suggesting an increase in metabolism, although $t_{½}$ is not appreciably reduced **(Table 4.3.4 b)**. Importantly, this moves the curve marginally below that of the PXR/CAR DHu, with no significant difference in parameters between these two models. Once again, the effect of humanizing PXR is dependent on CAR status. In the HuPXR/CAR KO model, there are marginal, but non-significant, changes in parameters relative to wild-type, with small increases in C_{max} and drug exposure, and small reductions in clearance and $t_{½}$. These changes yield a curve which is located slightly above that of the PXR/CAR DHu model. However, the significantly lower systemic exposure observed in the HuPXR/mCAR model in the 1 day population is maintained in the 3 day population, with significantly higher clearance and lower C_{max} being recorded, together with non-significant reductions in drug exposure and $t_{½}$. A similar situation is seen when CAR is humanized, with metabolism being strongly dependent on PXR status. There is no difference in metabolism in the PXR KO/HuCAR model, with the curve mirroring that of the wild-type model. However, systemic exposure is reduced in the mPXR/HuCAR model, with a significant increase in clearance being recorded, together with non-significant reductions in C_{max} and drug exposure.

Knocking-out both receptors influences the metabolism of anastrozole, with clearance being significantly increased and marginal reductions being recorded in C_{max} and drug exposure, although $t_{½}$ is not changed. There is no significant change in metabolism in the PXR KO/HuCAR model. However, although clearance is unchanged in the PXR KO/mCAR model, there are non-significant reductions in C_{max} and drug exposure, together with a notable increase in $t_{½}$. In contrast to the PXR KO models, the effect of knocking-out CAR appears to be species-specific. The profile derived from the mPXR/CAR KO model displays minimal differences to that of the wild-type, with no change in clearance, C_{max} and drug exposure, although there is a non-significant increase in $t_{½}$. In

contrast, although there are no significant changes in the HuPXR/CAR KO model, there is a trend towards higher systemic exposure, with increases in C_{max} and drug exposure, together with a reduction in clearance. There is also a slight reduction in $t_{½}$.

4.3.5 THE AROMATASE INHIBITORS INDUCE CYP2B6 IN THE rCYP2B6-LACZ REPORTER MOUSE

Expression of lacZ is induced in all models treated with aromatase inhibitors, although only two thirds of mice treated with letrozole demonstrate induction of the reporter gene, indicating inter-individual variation **(Figure 4.3.17 & 4.3.18)**. In all subjects in which induction is seen, the reporter is expressed in zone 2 of the liver acinus, and localised in the cytoplasm and nucleus of the hepatocytes **(Figure 4.3.19)**. Anastrozole induces a significantly stronger response than letrozole, and the magnitude of response is sexually dimorphic in the mPXR/mCAR background model, with lacZ expression being stronger in the males than in the females. However, magnitude of response is also significantly species-specific, with reporter expression in response to letrozole treatment being significantly lower in the human background than the mouse. Sexual dimorphism and species-specificity with respect to anastrozole treatment could not be assessed in the hPXR/hCAR background as a result of low mouse numbers. Few significant differences in biochemical markers can be seen, although there was significant intra-population variability in the majority of assays. However, significant increases in blood creatinine concentrations were observed in both males and females following letrozole therapy, and in females only following anastrozole treatment, on the murine PXR/CAR background **(Figure 4.3.20)**. No changes in creatinine concentrations were observed on the human PXR/CAR background.

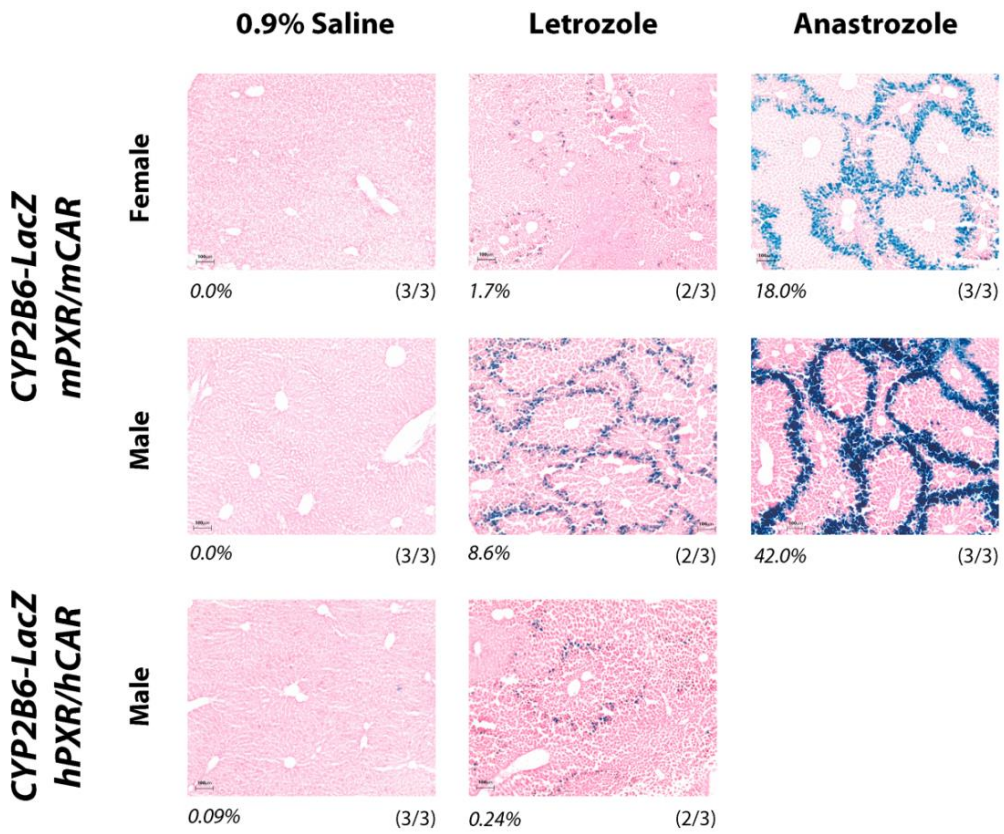


Figure 4.3.17: LacZ staining following 3 day drug treatment (10x magnification)

CYP2B6-LacZ (mPXR/mCAR; male and female) and CYP2B6-LacZ (hPXR/hCAR; male only) reporter mice (n=3 per group) were treated p.o. daily for 3 days with 0.9% Saline, 25 mg/kg Letrozole or 20 mg/kg Anastrozole. Livers were harvested into 1% PFA (w/v), cryofixed and sectioned before staining for nuclear fast red, followed by LacZ. Photos were taken at 10x magnification and are representative of the treatment group. Scales indicate 100mm. Numbers in brackets indicate number of group for which the photo is representative. Percentages indicate the percentage of cells exhibiting LacZ staining according to densitometry

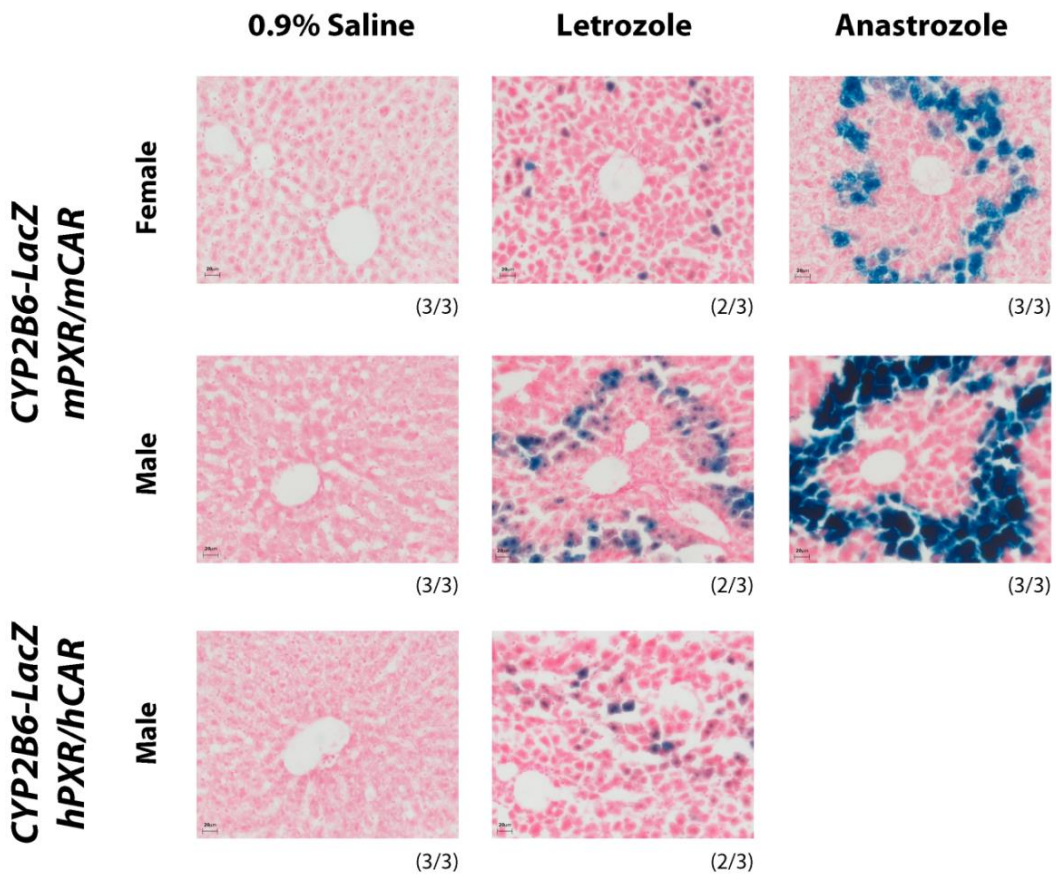


Figure 4.3.18: LacZ staining following 3 day drug treatment (40x magnification)

CYP2B6-LacZ (mPXR/mCAR; male and female) and CYP2B6-LacZ (hPXR/hCAR; male only) reporter mice (n=3 per group) were treated p.o. daily for 3 days with 0.9% Saline, 25 mg/kg Letrozole or 20 mg/kg Anastrozole. Livers were harvested into 1% PFA (w/v), cryofixed and sectioned before staining for nuclear fast red, followed by LacZ. Photos were taken at 40x magnification and are representative of the treatment group. Scales indicate 20mm. Numbers to lower right of photos indicate number of group for which the photo is representative.

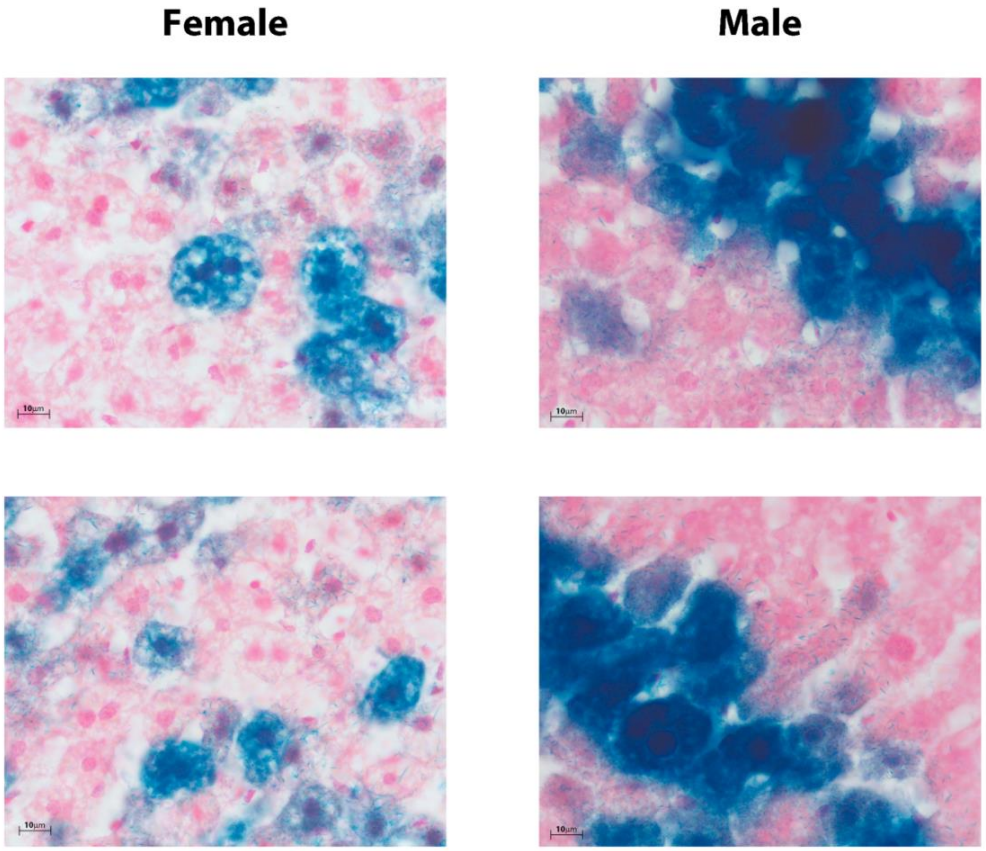


Figure 4.3.19: LacZ staining following 3 day treatment of CYP2B6-LacZ (mPXR/mCAR) mice with 20 mg/kg Anastrozole (100x magnification)

Male and female CYP2B6-LacZ (mPXR/mCAR) reporter mice (n=3 per group) were treated p.o. daily for 3 days with 20 mg/kg Anastrozole. Livers were harvested into 1% PFA (w/v), cryofixed and sectioned before staining for nuclear fast red, followed by LacZ. Two photos were taken from each section at 100x magnification and are representative of the treatment group. Scales indicate 10mm.

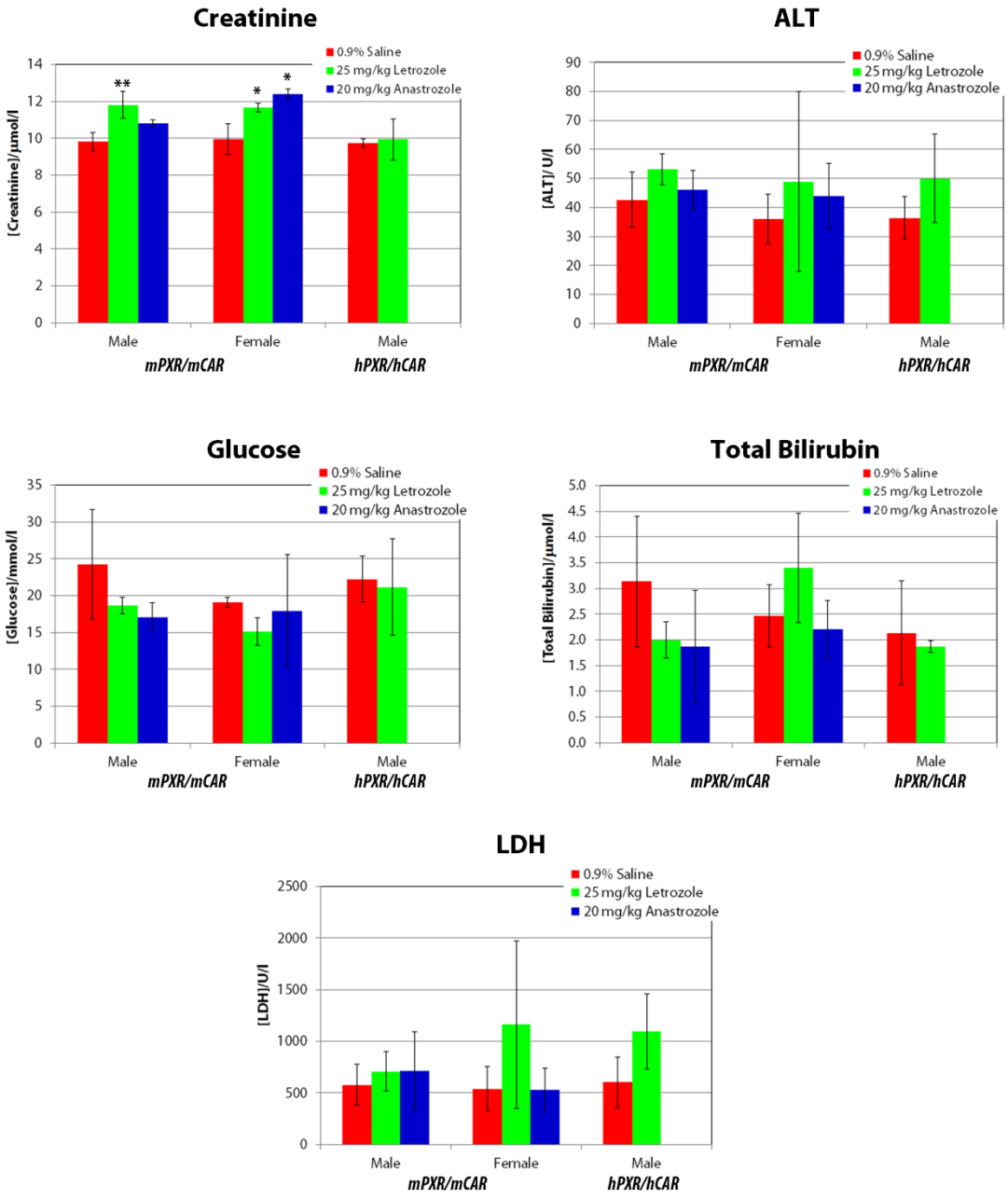


Figure 4.3.20: Biochemical blood marker analysis following 3 day drug treatment

*CYP2B6-LacZ (mPXR/mCAR; male and female) and CYP2B6-LacZ (hPXR/hCAR; male only) reporter mice (n=3 per group) were treated p.o. daily for 3 days with 0.9% Saline, 25 mg/kg Letrozole or 20 mg/kg Anastrozole (mouse background only). Serum was separated from heparinized whole blood by centrifugation and analysed for creatinine, ALT, glucose, total bilirubin and LDH concentrations at the Clinical Pathology Service, MRC Harwell. Graphs indicate average concentration ± S.D. Statistics analysed concentration following drug treatment vs vehicle control. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.*

4.4 DISCUSSION

In this chapter, the aromatase inhibitors are shown to induce P450s in a PXR- and CAR-specific manner, demonstrating species-specific and sex-specific cross-talk between these receptors, with PXR/CAR-mediated variations being observed in downstream pharmacokinetics. All data is summarized in **Tables 4.3.1 & 4.3.2**, with all relevant inductions/repressions indicated by arrows. It must also be noted that Western blot data is expressed relative to vehicle control, whereas data derived from fluorigenic P450 activity assays and PK analysis is expressed relative to wild-type data.

4.4.1 CYTOCHROME P450 EXPRESSION IS PXR/CAR DEPENDENT AND DEMONSTRATES SPECIES SPECIFICITY

Prior to analysis of drug pharmacokinetics, it was important to examine changes in the P450 expression profile to provide a potential basis for pharmacokinetic variations. The induction profile of several key P450s was therefore characterised using Western blot and fluorescent P450 assays. Only those enzymes displaying enzyme induction will be discussed in this section, and therefore no reference will be made to Cyp2c or POR, neither of which displayed significant induction.

4.4.1.1 Cyp1a

No variations were observed in Cyp1a protein expression or activity following treatment with either drug in males **(Figures 4.3.1, 4.3.3, 4.3.5 & 4.3.6)**. However, although there is no Cyp1a protein induction in females, Cyp1a activity is induced in various genotypes: 1) mPXR/HuCAR, HuPXR/CAR KO and PXR/CAR DHu following letrozole treatment, and 2) PXR KO/HuCAR, mPXR/HuCAR, HuPXR/CAR KO and PXR/CAR DHu following anastrozole

Sex	Genotype		Gene						Activity probe							PK parameter				
	PXR	CAR	Cyp 1a	Cyp 2a	Cyp 2b	Cyp 2c	Cyp 3a	POR	BFC *3A4*	BQ *3A4*	EFC *2B/ 2C*	MFC *2B/ 2C*	MR *1A2*	ER *1A1 /2*	BR *2B6*	$T_{½}$	C_{max}	CL	AUC 1	AUC 2
Male	M	M			0		↑ 1.5													
	KO	M			0								↓							
	KO	Hu			0						↓*		↓*	↓***				↓*		
	M	Hu			0		↑ 2.4		↑***	↑***	↑***		↓	↓**		↑***		↓		↑
	M	KO			0		↑ 1.8		↑***	↑***					↑				↑	↑
	Hu	KO			0		↑ 1.7	↑ 1.4						↓*						
	Hu	M			0			↑ 1.3					↓	↓***		↓*		↑*		
	KO	KO			0		↓ 0.6		↓	↓	↓**	↓	↓*	↓***	↓			↓*		
	Hu	Hu			0	↑ 1.5		↑ 1.6					↓	↓**		↓		↓*	↓	↓
Female	M	M																		
	KO	M			↑ 1.6								↑	↑	↑	↓	↓***	↑***	↓***	↓***
	KO	Hu														↓	↓***	↓*	↓***	↓***
	M	Hu			↑ 2.3	↑ 1.6	↑ 1.6	↑ 1.5	↑	↑**	↑***	↑***	↑*	↑*	↑***		↓***	↑	↓***	↓*
	M	KO			0										↑*	↓	↓		↓**	↓*
	Hu	KO			0		↑ 1.7	↑ 1.5					↑***	↑***	↑*	↓	↓**	↑*	↓***	↓***
	Hu	M														↓	↓**	↑**	↓***	↓***
	KO	KO			0											↓	↓**	↓	↓**	↓*
	Hu	Hu	↑ 1.5		↑ 1.9		↑ 1.9	↑ 2.0	↑	↑	↑	↑*	↑*	↑***	↑***	↓	↓***	↑***	↓***	↓***

Table 4.4.1: Summary of all data following 3 day letrozole treatment

Western blot data expressed relative to vehicle control, activity and PK data expressed relative to wild-type. *↑ = induced, ↓ = repressed. AUC 1 = AUC(0→1440), AUC 2 = AUC(0→∞). Statistics represent the comparison of transgenic data vs wild-type.* * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001. Red = >2-fold induction, Green = >2-fold repression, 0 = no expression. Superscript numbers indicate densitometry data relative to vehicle control (fold induction). Cyp2b and Cyp3a densitometry data refers to Cyp2b10 and Cyp3a11 isoforms only. Main isoform detected by P450 assay given in title.

Sex	Genotype		Gene						Activity probe							PK parameter				
	PXR	CAR	Cyp 1a	Cyp2a	Cyp 2b	Cyp 2c	Cyp 3a	POR	BFC *3A4*	BQ *3A4*	EFC *2B/ 2C*	MFC *2B/ 2C*	MR *1A2*	ER *1A1 /2*	BR *2B6*	$T_{½}$	C_{max}	CL	AUC 1	AUC 2
Male	M	M			↑1.7															
	KO	M			↑ 2.5				↑	↑	↓	↓				↑		↓**		
	KO	Hu			↑1.6							↓								
	M	Hu			↑1.8		↑3.3		↑***	↑***						↑				
	M	KO		↑1.6	0	↑1.7	↑ 2.0		↑***	↑***						↑	↑*	↓**	↑*	↑*
	Hu	KO			0		↑2.7		↑	↑	↓	↓			↓	↑		↓	↑	↑
	Hu	M			↑ 8.6				↑	↑	↑	↑*			↑		↓	↓***	↓	↓
	KO	KO		↓0.6	0				↓		↓	↓			↓	↑	↑*	↓	↑**	↑**
	Hu	Hu		↓0.7	↑ 2.0						↓	↓			↓		↑***	↓	↑	↑
Female	M	M			↑ 4.6															
	KO	M			↑ 6.1						↓	↓			↓	↑	↓	↓***	↓	↓
	KO	Hu			↑1.6		↑1.9		↑		↓	↓*	↑***	↑	↓					
	M	Hu		↑1.6	↑ 2.9		↑1.8		↑*	↑	↑		↑		↓		↓	↑*	↓	↓
	M	KO			0		↑1.6		↑					↓	↓	↑		↓***		
	Hu	KO			0						↓*	↓**	↑***		↓	↓	↑	↓	↑	↑
	Hu	M			↑ 5.1											↓	↓*	↑***	↓	↓
	KO	KO			0				↓		↓**	↓**		↓	↓		↓	↑*	↓	↓
	Hu	Hu		↑1.6	↑3.7	↑1.9	↑1.6	↑1.7			↓	↓	↑		↓					

Table 4.4.2: Summary of all data following 3 day anastrozole treatment

*Western blot data expressed relative to vehicle control, activity and PK data expressed relative to wild-type. ↑ = induced, ↓ = repressed. AUC 1 = AUC(0→1440), AUC 2 = AUC(0→∞). Statistics represent the comparison of transgenic data vs wild-type. * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001. Red = >2-fold induction, Green = >2-fold repression, 0 = no expression. Superscript numbers indicate densitometry data relative to vehicle control (fold induction). Cyp2b and Cyp3a densitometry data refers to Cyp2b10 and Cyp3a11 isoforms only. Main isoform detected by P450 assay given in title.*

treatment **(Figure 4.3.8)**. Notably, induction in Cyp1a activity is not observed in any model possessing mCAR, indicating a species-specific effect. It is highly likely that the isoform responsible for this increase in activity is the Cyp1a2 isoform, being the only member of the Cyp1 family of enzymes expressed in the liver in wild-type mice (Hrycay and Bandiera, 2009), and concurring with the observed increase in MR assay, which is more specific for the Cyp1a2 isoform **(Figure 4.3.8)**.

Although there are no specific genotype influences that can be identified following letrozole treatment, there is a stronger association between hCAR and increased Cyp1a activity following anastrozole treatment **(Figure 4.3.8)**. Although the Cyp1a genes are prototypically induced by the aryl hydrocarbon receptor (Nukaya et al., 2009), a recent study has described a novel transcriptional circuit associating CYP1A1 and CYP1A2 induction with CAR activation, in which CAR can both activate CYP1A expression and enhance aryl hydrocarbon receptor-induced activation (Yoshinari et al., 2010). It is also gender specific, with the increased activity appearing in females only, in contrast to previously published data that suggests CYP1A2 activity is higher in males (Schwartz, 2007; Waxman and Holloway, 2009). However, this observation does give further corroboration to the involvement of CAR in the induction of CYP1A, with literature suggesting that CAR is more active and more highly expressed in females than males (Forman et al., 1998; Lamba et al., 2003; Petrick and Klaassen, 2007; Hernandez et al., 2009b).

4.4.1.2 Cyp2a

No variations were seen in Cyp2a protein expression following letrozole treatment in either sex, although the female bias in constitutive expression previously reported was observed (Hernandez et al., 2006; Hrycay and Bandiera, 2009). This could have significant repercussions in terms of drug pharmacokinetics, because it suggests that letrozole metabolism could be slower in males than females as a result of the lower constitutive expression of the Cyp2a enzymes. However, because no activity data is available for this

enzyme group, it is impossible to assess what the downstream effects on pharmacokinetics will be.

There was also no significant variation in Cyp2a in males following anastrozole treatment. However, in females marginal induction of Cyp2a enzymes is observed in those genotypes possessing the hCAR moiety. This concurs with the role of CAR as a key regulator of Cyp2a4 and Cyp2a5, two of the most important members of the Cyp2a family in mice, as well as the two members demonstrating sex-specific regulation (Wei et al., 2002; Honkakoski et al., 2003; Hernandez et al., 2006; Hrycay and Bandiera, 2009). This effect is species-specific, with no response observed in mice possessing native CAR. It is also gender-specific, again as a probable result of the higher expression and activity of CAR in mice. These data add further evidence to the role of anastrozole as an activator of hCAR.

4.4.1.3 Cyp2b

Cyp2b10 expression and activity is induced in response to treatment with letrozole and anastrozole in a PXR/CAR- and gender-dependent manner **(Figure 4.3.1-4.3.8)**. Expression is seen only in those models possessing a functional variant of CAR, thus demonstrating its key role in the regulation of Cyp2b10. The regulation of Cyp2b10 by CAR is also gender-specific in nature, showing higher CAR-related basal expression and induction in females than males, concurring with previous reports (Hrycay and Bandiera, 2009). This can be attributed to the higher expression and activity of both Cyp2b10 and CAR in females (Jarukamjorn et al., 2002; Lamba et al., 2003; Petrick and Klaassen, 2007; Hernandez et al., 2009b). Cyp2b10 is induced to a significantly greater extent following anastrozole treatment than letrozole. Although basal and induced expression is seen in all CAR models in females following letrozole treatment, not only is there no induction of Cyp2b10, there is no expression in any model in the male cohort. However, Cyp2b10 is induced in all models carrying a functional CAR moiety following anastrozole treatment, regardless of gender, although the latter still displays stronger basal expression and

significantly stronger induction than the males. Protein expression and activity data concur in most groups. However, the extent of induction does not mirror that predicted from protein expression data in females treated with anastrozole.

The specificity of these drugs in inducing Cyp2b10 suggests that both are likely to interact with CAR, the main regulator of Cyp2b10, with anastrozole being the stronger inducer. This hypothesis is given further credence by the lack of Cyp2b10 protein and enzyme activity induction in all models in which CAR is knocked-out, as well as the identification of modest CAR differential variations in Cyp1a and Cyp2a proteins following anastrozole treatment. It is impossible to assess whether these drugs bind directly to CAR or act indirectly from this data. However, given that the majority of identified CAR modulators act indirectly, and that the magnitude of response is significantly smaller than observed following treatment with known prototypical CAR ligands (Scheer et al., 2008; Ross et al., 2010), it is probable that the aromatase inhibitors are indirect modulators of CAR activity. Also, because the aromatase inhibitors modulate sex-hormone levels, it is likely that any change in CAR activation is likely to be enhanced, if not caused, by the pharmacological action of these drugs. It is well documented that 17β-oestradiol and oestrone both activate mCAR by enhancing the interaction of mCAR with co-activators, such as SRC-1, and co-repressors, such as NCoR, following xenobiotic treatment (Kawamoto et al., 2000; Mäkinen et al., 2003; Min, 2010; Dau et al., 2012). The ability of oestradiol to activate hCAR and synergistically induce CYP2B6 with the activated oestrogen receptor at high concentrations has also been recently reported (Koh et al., 2012). Various androgens have also been shown to repress CAR activity (Forman et al., 1998; Gillberg et al., 2006; Hernandez et al., 2009b; Monostory and Dvorak, 2011). However, the lack of data regarding circulating oestrogen concentrations in this study, together with the lack of mechanistic studies regarding CAR activation by aromatase inhibitors, prevents any conclusions being drawn regarding the regulation of CAR activity.

There also appears to be significant species-specificity in the magnitude of response related to PXR/CAR crosstalk which is particularly evident when

examining the anastrozole treated cohorts as a result of the higher induction levels observed. Firstly, the presence of hCAR induces Cyp2b10 to a similar extent to that seen in the wild-type model, regardless of PXR genotype. This indicates that hCAR activation alone is sufficient to achieve induction of this protein. However, induction as a result of mCAR activation is strongly affected by PXR genotype, being higher than wild-type when mCAR is combined with hPXR. There is also an induction in Cyp2b10 relative to wild-type when mCAR is present on the PXR KO background, although this is gender-specific, being significant in females but marginal in males. These data are also reflected in the Cyp2b10 activity assays. This suggests two possibilities in terms of crosstalk between these receptor variants. Firstly, mPXR constrains the ability of mCAR to induce Cyp2b10 expression, as illustrated by the increased protein expression recorded following anastrozole treatment in the PXR KO/mCAR model, with gender specificity likely to be as a result of the higher activity of CAR in females. Secondly, mCAR synergizes with hPXR to achieve maximal induction following anastrozole treatment.

4.4.1.4 Cyp3a

Cyp3a11 is also induced in response to AI treatment, and is subject to species-dependent regulatory crosstalk between PXR and CAR. Being dependent on other nuclear receptors, such as hepatocyte nuclear factor 4α, for its constitutive expression, Cyp3a11 is visible in all models. Also in agreement with human expression data, this gene is more highly expressed in females than males (Hunt et al., 1992; Down et al., 2007; Greenblatt and Von Moltke, 2008; Hu and Zhao, 2010).

Sexual dimorphism is a key feature of the control of Cyp3a11 by PXR/CAR in response to AI treatment. In males, both AIs induce Cyp3a11 expression in the HuPXR/CAR KO and two mPXR models, with the highest induction recorded in the mPXR/HuCAR model **(Figures 4.3.1 & 4.3.3)**. There is also a marginal repression in response to both treatments in the PXR/CAR DKO model. These findings concur with data from the Cyp3a activity assays (BFC and BQ), in which

the highest activity is detected in the mPXR models **(Figure 4.3.5)**. This suggests that the impact of AI treatment on the expression of Cyp3a11 in males is regulated by a common, species-dependent PXR/CAR-related mechanism. The data indicates that the primary regulator of AI induced Cyp3a11 expression in males is PXR, as demonstrated by the induction observed in both CAR KO models, the lack of induction in PXR KO models and the repression observed in the PXR/CAR DKO model, a finding in keeping with the prototypical role of PXR in Cyp3a11 induction (Quattrochi and Guzelian, 2001; Stanley et al., 2006; di Masi et al., 2009). These data also suggest that activation of mPXR in response to treatment is sufficient to elicit a Cyp3a11 response, regardless of CAR status, although mPXR and hCAR do appear to synergize to achieve maximal induction. This is in contrast to hPXR-mediated regulation, in which upregulation in response to activation is strongly related to CAR status, occurring only in the CAR KO model, indicating that both mCAR and hCAR interact with hPXR to repress Cyp3a11 induction.

In females, the induction of Cyp3a11 in response to AI therapy is significantly smaller than that observed in males, although basal expression is greater **(Figures 4.3.2 & 4.3.4)**. The only common observation between the two drug treatments is a marginal induction in the PXR/CAR DHu model, which is greater in the letrozole treated cohort, although still less than 2-fold in both groups. There is also a slight induction in the mPXR/HuCAR model that translates to an approximately 2-fold induction in enzyme activity in the BFC and BQ assays. No other significant changes in Cyp3a11 expression can be seen following letrozole treatment. Stronger induction, reflected in the activity assays, is observed in the female anastrozole cohort **(Figures 4.3.4 & 4.3.7)**. As recorded in the males, induction is observed in both mPXR models, with the mPXR/HuCAR model demonstrating the stronger effect. However, the strongest induction in Cyp3a11 protein expression, although not the highest activity, was recorded in the PXR KO/HuCAR model. These observations suggest that although mPXR is associated with Cyp3a11 regulation, and particularly with maximal enzyme activity, there is also a strong hCAR element involved in anastrozole-mediated induction. Although surprising given the data obtained from the male cohort,

these observations are not without precedent, since Cyp3a11 has also been identified as a CAR target gene, upregulated in response to CAR ligands, such as nonylphenol and TCPOBOP (Wei et al., 2002; Wagner et al., 2005; Down et al., 2007; Hernandez et al., 2009b). This hypothesis also corroborates observations gathered from the Cyp1a, Cyp2a and Cyp2b10 genes, all of which suggest that anastrozole is an hCAR activator. mCAR appears to have a repressive effect on Cyp3a11 induction, with no variation being observed in any model possessing this moiety. Also, except in the presence of hCAR, hPXR has no effect on Cyp3a11 expression, indicating that anastrozole activates mPXR only.

The repression identified in the PXR/CAR DKO model in both male treatment groups is also observed in the female letrozole group. Unfortunately, as a result of poor band separation in the female anastrozole cohort, it is impossible to identify any repression in Cyp3a11 for this treatment group. However, from the data available, it appears that this effect is sex-independent. It is likely that the repression in Cyp3a11 expression recorded in the PXR/CAR DKO model in response to both drugs is as a result of drug action on one of the constitutive regulators of basal protein expression, such as HNF-4α (Kamiya et al., 2003; Wiwi et al., 2004; Inoue et al., 2011). However, the dissection of this effect is outwith the scope of this study.

Once again, the ability of these drugs to directly bind to PXR cannot be ascertained from these data, although it is more likely given the highly promiscuous nature of the PXR ligand binding domain. However, it is also probable that there will be some indirect modulation of PXR activity as a result of the pharmacological action of these drugs. hPXR has previously been shown to be activated by oestrone and oestradiol, suggesting that overall PXR activity , and thus Cyp3a11 expression, is likely to be reduced in those mice treated with AIs (Mnif et al., 2007; di Masi et al., 2009). A recent study has also described an inhibitory crosstalk mechanism between PXR and the androgen receptor, in which both receptors inhibit the activity of the other, a mechanism of importance when considering the male population (Kumar et al., 2010). This aspect of PXR control is of importance when considering response to endocrine modulators, and therefore requires further analysis. Unfortunately, the data

generated in this study is insufficient to test this hypothesis, and therefore this would be a task for future investigation. However, assuming that PXR activity is repressed in response to the hormonal changes that characterize the clinical response to AI therapy, these data indicate that AIs activate PXR, either directly or indirectly.

4.4.2 Letrozole pharmacokinetics are PXR/CAR dependent, but not influenced by Cytochrome P450 expression

Although PXR/CAR status does impact on drug clearance, the small clearance values returned by this study relative to the rate of hepatic blood flow in mice (90 ml/min/kg; Davies *et al.*, 1993) suggest that the predominant route of letrozole excretion could be renal. Maximal renal clearance is defined by the glomerular filtration rate in mice, which is given as 14 ml/min/kg (Davies and Morris, 1993). It is evident from the study data that clearance is significantly lower than this in our models, and that it varies according to PXR/CAR genotype, although further studies are required to confirm the role of renal elimination in murine letrozole disposition. If renal elimination is proved, it is highly unlikely that variations in P450 expression and activity in the liver will have a significant effect on letrozole clearance in these models, with the most likely mediators of this effect being the drug transporters. Both PXR and CAR have been associated with the regulation of various membrane bound drug transporter proteins expressed in the kidney, liver and enterocytes including Mdr1, Mrp1-6 and various OATP transporters (Klaassen and Aleksunes, 2010). This discussion will therefore focus on potential perturbations in these proteins, although reference will be made to P450 expression if deemed relevant.

4.4.2.1 LETROZOLE PHARMACOKINETICS ARE SPECIES-SPECIFIC WITH RESPECT TO PXR AND CAR

To assess the impact of PXR and CAR on letrozole pharmacokinetics, the 72 hour data were analysed **(Figure 4.3.10 & 4.3.12, Tables 4.3.1b & 4.3.2b)**. An important feature common to both sexes is that letrozole pharmacokinetics are altered in the PXR/CAR DHu model relative to wild-type, with drug being cleared significantly faster in this model, and especially in females. This sexual dimorphism is driven entirely by the sex-dependent pharmacokinetics demonstrated by the wild-type model, in which females demonstrate slower elimination than males. There is no variation in letrozole pharmacokinetics in the PXR/CAR DHu model between males and females. This therefore indicates that the human receptors have a greater activity in response to letrozole treatment than the murine, and also that murine receptor activity is subject to sexually dimorphic control.

Isolating murine and human CAR indicates that there is no species-specific variation in activity between these two moieties, with both producing profiles most closely resembling those of the PXR/CAR DHu model. This indicates that CAR activation by letrozole in the absence of PXR is sufficient to elicit faster drug clearance. In contrast, PXR exerts a predominantly repressive effect on letrozole clearance when working in isolation. mPXR exerts a significant repressive influence on mCAR activity in the wild-type model, a hypothesis given further credence by the observation that pharmacokinetics in the mPXR/CAR KO and mPXR/HuCAR models most closely resemble those of the wild-type. It is therefore suggestive that mPXR is the driving factor underlying pharmacokinetics in these models. In contrast, although the profile of the HuPXR/CAR KO model is most closely related to that of the wild-type, it is clear that synergism with CAR, regardless of species, results in high letrozole clearance, with synergy between the human receptors inducing the greatest increase.

Earlier protein analysis indicated that PXR and CAR interact in a species-specific manner to control P450 induction and activity in response to letrozole.

However, when examining the pharmacokinetic data, there is little conformity with P450 expression trends. A good example of this disparity is that of the mPXR/HuCAR model, in which Cyp3a11 was most strongly induced in response to letrozole treatment, combined with a marginal induction in the expression of Cyp2a in females only, and therefore faster metabolism may have been expected. However, pharmacokinetic analysis demonstrates reduced elimination in both sexes relative to wild-type. This is strongly suggestive of an alternative elimination route, such as renal clearance, and thus implicates other elements of the drug metabolism pathway, such as drug transporters. One possible exception to the disparity between P450 induction and pharmacokinetics is the observation that mPXR constrains mCAR-mediated Cyp2b10 induction, a finding concurrent with the pharmacokinetic data. To date, no analysis has identified a role for CYP2B6 in letrozole metabolism. However, no systematic analysis of letrozole metabolism in mice is available in the literature, and therefore it is possible that this enzyme mediates letrozole metabolism in this species, thus providing a rationale for the pharmacokinetics observed in the wild-type.

Further evidence for an alternative elimination route is provided by the PXR/CAR DKO model, in which elimination occurs at a rate between that of the wild-type and PXR/CAR DHu models. This indicates that neither PXR nor CAR are obligate for letrozole elimination in mouse, although data from the other models demonstrates that PXR/CAR-mediated modulation does occur. Interestingly, gender-differential elimination is also seen in this model, being faster in males than females. Once again, these observations do not equate to data from the Western blots, in which repression of Cyp3a11 and Cyp2a are stronger in the males, which would suggest that metabolism is likely to be slower in this cohort if P450 related. This further corroborates the theory that P450 metabolism plays a minor role in letrozole disposition in mice.

These data clearly demonstrate that letrozole pharmacokinetics are modulated by PXR/CAR, although neither are obligate. CAR activation is required to achieve maximum clearance in mice, regardless of species. However, the magnitude of elimination is strongly associated with species-specific PXR

interaction, with mPXR repressing CAR activity whilst hPXR maintains/promotes activity observed in the PXR KO models, both regardless of species of CAR. It is therefore evident that letrozole interactions with PXR are strongly species-dependent. Gender-differential pharmacokinetics are also observed in both the wild-type and PXR/CAR DKO models. Significant gender-related influences on the metabolism and pharmacokinetics of letrozole in rats have been previously described (Liu et al., 2000; Tao et al., 2007; Wempe et al., 2007), demonstrating the importance of considering gender in pre-clinical drug assessment.

Further study is required to establish the mechanisms underlying both species- and gender-differential variations in pharmacokinetics. It is also of importance to identify the regulator involved in the control of letrozole clearance in the PXR/CAR DKO model, as well as to perform a full analysis of letrozole metabolism in mice to establish the role of Cyp2b10 in letrozole disposition. Unfortunately, comparison between this data and that previously generated is not possible because no pre-clinical data regarding letrozole pharmacokinetics in mice is available in the literature, with data regarding metabolism in other species, such as rats, being minimal. This study is therefore the first to describe gender- and species-specific clearance of this drug in mice.

4.4.2.2 PHARMACOKINETIC VARIATIONS ARE PROBABLY A RESULT OF DRUG TRANSPORTER MODULATION

The previous section was primarily concerned with the clearance parameter, possibly the most important to pharmacokinetics because it is a measure of the ability of the body to permanently eliminate a drug (Toutain and Bousquet-Mélou, 2004b). However, as a result of the oral dosing mechanism employed, clearance data generated by non-compartmental modelling actually represents clearance ÷ bioavailability, and therefore can be influenced by any factor that impacts bioavailability, in addition to those that affect clearance itself. This therefore suggests three potential explanations for the variations observed in these data, all of which are related to the expression of drug efflux transporters:

1) Letrozole bioavailability varies with PXR/CAR genotype; 2) Tissue distribution varies with PXR/CAR genotype; and 3) Differential renal clearance is regulated by PXR/CAR.

Bioavailability is defined as the extent and rate at which a drug or active moiety is extracted from its pharmaceutical formulation and becomes available to the general circulation (Toutain and Bousquet-Mélou, 2004a). As such, it can be influenced by the physicochemical properties of the drug itself, such as pK_a and lipophilicity, by physiological factors, such as intestinal transit time and absorption mechanism, and also by the effects of first pass metabolism (Song et al., 2004). It is most likely that any PXR/CAR related variation in bioavailability will be as a result of alterations in first-pass metabolism or the absorption of drug by transporter proteins. Previous studies have indicated that first pass metabolism in the intestine and liver are of limited importance in the distribution of letrozole, as indicated by the high bioavailability described for this drug in humans (Sioufi et al., 1997a). Study data concurs with the limited role of hepatic metabolism in the disposition of this drug. However, in order to exclude intestinal metabolism of letrozole as a potential source of variation in bioavailability, expression and activity analysis must be performed using small intestinal microsomes, such as was performed on the liver samples.

The most important potential source of variation in bioavailability lies in the expression of the drug transporters by the enterocytes, which can influence both drug entry into the circulation and increase the opportunity for intestinal first-pass metabolism by increasing drug efflux into the intestinal lumen (Nicolas et al., 2009; Klaassen and Aleksunes, 2010). This has already been demonstrated to be of importance to letrozole disposition in a study in rats, and, with oral bioavailability being significantly lower in males than females (≈ 38% vs ≈ 95%), and terminal half-life significantly slower in females, as a result of variations in letrozole absorption. Of concern is the potential role of the drug-efflux transporters located at the apical surface of the enterocytes in reducing letrozole uptake into the circulation, which include Mrp2, Bcrp and P-glycoprotein, all of which are targets of PXR/CAR (Klaassen and Aleksunes, 2010). Of particular importance to this study is the identification of P-

glycoprotein as a primary downstream target of PXR activation, although recent evidence also implicates CAR activation in its regulation (Burk et al., 2005; Teng and Piquette-Miller, 2008; Bebawy and Chetty, 2009; Harmsen et al., 2010; Klaassen and Aleksunes, 2010; Wang et al., 2010; Chan et al., 2011; Chen et al., 2012). Any interaction between letrozole, this transporter and PXR/CAR could therefore result in significantly altered bioavailability.

Another hypothesis is that the level of drug distribution in the body is variable, resulting in changes to the quantity of drug available to the circulation for elimination. An indication of altered tissue distribution can be obtained from the analysis of apparent volume of distribution (V_d), a parameter that can be derived from $t_{½}$, although it cannot be used diagnostically for this purpose without further direct information regarding intra-tissue drug concentrations (Toutain and Bousquet-Mélou, 2004c). Variations in this parameter can indicate changes in tissue distribution and bioavailability, both of which rely on the expression and activity of drug transporters in various tissues. P-glycoprotein is expressed in numerous different tissues, including lungs, intestine and brain in mouse, and more extensively in human tissues (Klaassen and Aleksunes, 2010; Chen et al., 2012). It has a vital role in tissue distribution, protecting tissues from chemical injury by ejecting drug from the tissue into the circulation, and consequently changing V_d. The mPXR/HuCAR model is particularly interesting from this perspective, demonstrating a high $t_{½}$ associated with a marginally altered clearance relative to wild-type. It is also likely that volume of distribution is larger in the females then the males, an expected finding given the higher fat content of the female contingent, together with the lipophilicity of letrozole.

Given the main route of excretion, as suggested by clearance, the simplest explanation for the variations observed is that drug transporters in the proximal renal tubules are differentially modulated in response to letrozole. Various transporters have been identified in the kidneys, including P-glycoprotein, Mrp2, Mrp4 and BCRP on the apical membrane, all of which have been implicated in excreting drug into the proximal tubules, and all of which are regulated by PXR and CAR in the liver (Klaassen and Aleksunes, 2010; Chen et

al., 2012). Data regarding the regulation of these proteins in the kidney is very limited, although a recent study has identified PXR as a potential regulator of renal efflux transporters in type I diabetes and pregnancy mouse models (Yacovino and Aleksunes, 2012). Further analysis is required to characterise the control of renal efflux transporters, in addition to a full assessment of the role of renal clearance in letrozole elimination in mice, before any conclusions regarding the role of PXR and CAR can be assessed.

It is highly likely that the actual explanation will encompass aspects of all three hypotheses, given that all theories centre on the PXR/CAR-mediated differential regulation of transporters. However, the data set gathered by this study is insufficient to provide any firm evidence to support these hypotheses. Numerous experiments are therefore required to establish the role of drug transporters in the disposition of letrozole in mice. These include bioavailability studies for letrozole in each of the models tested, combined with tissue analysis for drug transporter expression and activity, and a closer examination of the expression and activity of intestinal P450 enzymes to rule out first-pass metabolism effects. An ideal study should also include the measurement of faecal and urinary excretion, as well as employing a detection method that can detect letrozole and its metabolites in plasma. The combination of these data streams will provide a robust basis on which conclusions regarding letrozole metabolism can be generated.

4.4.2.3 LETROZOLE PHARMACOKINETICS DEMONSTRATE TEMPORAL VARIATION IN A SEXUALLY DIMORPHIC AND SPECIES-SPECIFIC MANNER

When contrasting data from the 1 day cohort with that from the 3 day, it is clear that temporal variation in these models is highly dependent on gender, as well as on PXR/CAR genotype **(Figures 4.3.9-4.3.12, Tables 4.3.1 & 4.3.2)**. Examining males, it is noteworthy that there is no temporal variation in pharmacokinetics in the two PXR KO models, and only a marginal change in half-life in the PXR/CAR DKO model, indicating that temporal changes in pharmacokinetics in males are driven by PXR. PXR genotype also appears to be

important to temporal pharmacokinetic changes in females, with no variations observed in the PXR KO/HuCAR and PXR/CAR DKO models. However, drug elimination is increased by approximately 50% relative to one day cohort in the PXR KO/mCAR model, suggesting that mCAR has greater activity to effect temporal changes in the female than the male.

Interesting trends can also be developed from the comparison of temporal changes in wild-type and PXR/CAR DHu pharmacokinetics, which is also associated with significant gender-specificity. In the wild-type model, pharmacokinetic parameters indicate drug accumulation between 1 day and 3 day cohorts, which is marginal in males but notable in females, a finding consistent with data suggesting that letrozole is not completely eliminated from the bloodstream in this model within 24 hours. In contrast to wild-type data, a significant temporal change in pharmacokinetics is observed in the male PXR/CAR DHu model, with faster drug elimination and lower systemic exposure being apparent, but only marginal variation is observed in the females. This could indicate a gender-specific variation in bioavailability, as reported by Wempe *et al.* (2007) in rats. With several transporters having been reported as being expressed in a sexually dimorphic manner in intestine (Klaassen and Aleksunes, 2010), this is a theory that requires closer scrutiny. This could also be a result of transporter induction leading to faster renal clearance. If so, it suggests that transporter regulation varies between wild-type and PXR/CAR DHu models, as well as between sexes, a factor that has yet to be characterised in this model panel.

Perhaps the most extreme temporal variation was seen in the male mPXR/HuCAR model, in which higher systemic exposure and slower drug elimination are recorded. This variation is male-specific, with female pharmacokinetics showing little temporal variation. Although a marginal increase in drug exposure and reduction in clearance is a temporal feature of mPXR/CAR KO pharmacokinetics, changes on the scale observed in the mPXR/HuCAR model are specific to this genotype and suggest cross-talk between these receptors leading to aberrant target gene regulation.

Finally, HuPXR single transgenic models demonstrate little temporal variation in females. However, in males the temporal variation is profound, and is also CAR status dependent. Both models demonstrate reduced drug exposure. However, although clearance is marginally increased in the HuPXR/CAR KO model between the two time points, it is increased to a similar level to that of the PXR/CAR DHu model in the HuPXR/mCAR model, indicating that co-operative regulation by HuPXR and CAR is required for temporal variation in males.

From this analysis, it is clear that temporal variation in letrozole pharmacokinetics is a feature of all PXR genotypes in males, although species of PXR and CAR status are important when assessing the magnitude of variation. As previously discussed, pharmacokinetic changes in these models are likely to be as a result of species- and gender-specific variations in transporter activity, with many systemic drug transporters having been identified as expressed in a gender-specific manner (Morris et al., 2003; Klaassen and Aleksunes, 2010). To date, a systematic analysis of transporter localization, expression and activity in this model panel, examining both sexes, has not been published. However, this data indicates that it is likely to be differential with respect to PXR/CAR genotype and gender, and therefore this analysis should be performed as a matter of urgency to enable a full assessment of drug disposition, and to aid in the drawing of conclusions regarding PXR/CAR dependent regulation of non-hepatic drug disposition. However, it is also important to note that the P450-mediated metabolism cannot be completely excluded until a full analysis of letrozole metabolism in mice is performed.

4.4.3 Anastrozole pharmacokinetics are dependent on PXR and CAR status, with a greater dependence on hepatic clearance than letrozole

It is clear from a rudimentary examination of pharmacokinetic data that anastrozole disposition is significantly associated with PXR/CAR genotype. As

with letrozole, clearance data obtained following anastrozole treatment suggests that the primary route of elimination in many of the test models could be renal, having a clearance well below the glomerular filtration rate, although further analysis is required to confirm this hypothesis. However, unlike letrozole, pharmacokinetic data also indicates that hepatic metabolism is of relevance in several models, and particularly in the female cohorts, with clearance being greater than the glomerular filtration rate, although still significantly lower than the rate of hepatic blood flow. Anastrozole pharmacokinetics in these models will therefore be discussed with reference to protein expression and activity, although other hypotheses will also be postulated.

4.4.3.1 ANASTROZOLE PHARMACOKINETICS ARE PXR/CAR DEPENDENT AND DEMONSTRATE GENDER SPECIFICITY

A cursory analysis of the 72 hour pharmacokinetic parameter data for both sexes clearly indicates that anastrozole pharmacokinetics are differentially modulated according to PXR/CAR genotype in both sexes **(Figures 4.3.14 & 4.3.16, Tables 4.3.3b & 4.3.4b)**. However, equally clear is that these pharmacokinetics show significant gender variation, with a good rule of thumb being that males display lower drug elimination and higher drug exposure than the females.

A key example of both conclusions is demonstrated by the comparison of pharmacokinetics in the wild-type and PXR/CAR DHu models. Wild-type pharmacokinetics, whilst displaying sexual dimorphism, also illustrate one of the exceptions to the generalization given above, with higher clearance, faster drug elimination and lower drug exposure in the males than in the females. These changes do not equate to the variations recorded in P450 expression, suggesting that P450-mediated metabolism has no influence on anastrozole pharmacokinetics in this model. This concurs with the low clearance value which indicates a potentially more significant role for renal clearance in this model, and thus implicates transporter expression as a potential mediator of

inter-gender variability. Low clearance values are also observed in the PXR/CAR DHu model. However, whereas the PK profile in females is predominantly the same as that of the wild-type, indicating little variation in drug elimination between the two models, the profile in males is substantially different, demonstrating reduced clearance, slower elimination and increased drug exposure. Once again, P450 data does not equate to the observed changes in pharmacokinetics, suggesting that PXR/CAR mediated modulation is mediated by another entity, such as the drug transporters or UGTs. This clearly demonstrates not only that murine PXR/CAR and human PXR/CAR interact to regulate anastrozole elimination in a species-specific manner, but also that species-differential regulation is dependent on gender.

Gender variation is also evident when isolating the effects of each receptor. mPXR interacts with CAR to promote anastrozole clearance in a gender specific manner. In females, the profiles indicate that there is limited variation in pharmacokinetics between the wild-type and mPXR/CAR KO model, suggesting that mPXR drives wild-type pharmacokinetics in females. However, receptor synergy in the mPXR/HuCAR species hybrid model promotes a significant increase in clearance, indicative of faster elimination and consistent with the induction in Cyp3a enzyme activity observed in this model, a key element in hepatic anastrozole metabolism (**Figure 4.1.2**). In contrast, anastrozole clearance in males is maintained at wild-type levels in the presence of CAR, regardless of species. However, when mPXR is isolated from CAR, clearance reduces by approximately 50% and is associated with a significant increase in drug exposure, indicating reduced drug elimination. This is surprising when concurrently analysing pharmacokinetic data with that from the activity assays, in which anastrozole induces Cyp3a activity in the mPXR/CAR KO model, which would suggest an increase in metabolism, and thus clearance.

Gender- and species-specific metabolism is also observed when examining the HuPXR models. Isolating hPXR in males results in a reduced elimination in comparison to wild-type, although not as marked as that observed in the PXR/CAR DHu model, indicating a negative synergistic interaction between the human receptors. In females, a similar reduction in clearance is observed

relative to wild-type when hPXR is isolated. However, in contrast to the males, synergistic functioning between the two human receptors results in wild-type level clearance. One common feature between sexes is observed in the HuPXR/mCAR model, in which clearance is very significantly increased relative to wild-type, although this is stronger in males than females. However, none of these traits concur with observed Cyp3a activity profiles, indicating either that the increased elimination is via a non-hepatic (renal) route, or possibly that the UGT mediated N-glucuronidation pathway has a greater role in metabolism in the HuPXR/mCAR model.

Although PXR/CAR genotype does cause differential modulation of anastrozole pharmacokinetics, the analysis of the PXR/CAR DKO model demonstrates that neither receptor is essential for anastrozole metabolism. They also demonstrate that the moiety causing pharmacokinetic variation in this model acts in a sexually dimorphic manner, with the profiles in males and females demonstrating opposing effects. In males, clearance is markedly reduced relative to wild-type, yielding the lowest level of any model tested, and is associated with a significant increase in drug exposure. However, the parameter profile shows similarities to that of the PXR/CAR DHu model, suggesting that the interaction between hPXR and hCAR could be non-functional in males, and thus that pharmacokinetics in this model actually reflect control by another regulatory protein, such as the AhR, HNF-1α or HNF-4α. In females, the data suggests that PXR/CAR function could repress anastrozole elimination in all models excepting the two species-hybrid genotypes. This is demonstrated by an increased clearance and decreased drug exposure in the PXR/CAR DKO model relative to wild-type, yielding a profile resembling that of the mPXR/HuCAR model. Once again, it raises the question as to whether the similarity in profile indicates that the interaction between mPXR and hCAR is non-functional, and therefore that the pharmacokinetics observed in this model reflect an alternative regulatory pathway.

In summary, these data suggest that PXR and CAR are involved in the regulation of anastrozole elimination, although data from the PXR/CAR DKO model demonstrates that they are not essential. The role of PXR is both gender- and

species-specific. mPXR has stronger activity with respect to anastrozole disposition in females than males, being able to maintain wild-type clearance levels in the absence of CAR in contrast to the males. The species of CAR is of limited relevance with respect to mPXR activity, with the only notable variation being hCAR exerting a female-specific induction in clearance when working in conjunction with mPXR. However, species of CAR is of greater significance with respect to hPXR, with mCAR synergizing with hPXR in a gender-independent manner to promote the highest clearance recorded in any model. In contrast, hCAR interacts with hPXR in a gender-dependent manner, resulting in reduced clearance in males, but enhanced clearance in females relative to the HuPXR/CAR KO model. Interestingly, CAR acting in isolation promotes wild-type level clearance in both sexes, regardless of species, and therefore suggests that gender-differential pharmacokinetics are a direct result of interactions between CAR and PXR. It also demonstrates that Cyp3a activity alone is insufficient to explain variations in anastrozole pharmacokinetics, and provides further evidence that a non-P450 mediated elimination route, such as renal clearance or UGT-mediated N-glucuronidation, is responsible for the elimination of anastrozole.

These hypotheses cannot be assessed without further analysis, including investigating metabolite production profiles in these mice. It must also be remembered that a systematic analysis of anastrozole metabolism in mice has not been performed, and therefore potential inter-species variations in metabolism must be considered. Although of no physiological or clinical relevance, the identification of abnormally enhanced elimination in the two species hybrid models also provides a caution against using single PXR/CAR humanized mouse models in isolation to draw conclusions, requiring further confirmation from models with a greater clinical relevance to control for aberrant inter-species regulatory crosstalk.

4.4.3.2 ANASTROZOLE PHARMACOKINETICS ARE LIKELY TO BE INFLUENCED BY DRUG TRANSPORTER AND UDP-GLUCURONOSYLTRANSFERASE ACTIVITY

To this point, this study has demonstrated that PXR/CAR genotype does impact on anastrozole pharmacokinetics. However, pharmacokinetic analysis has revealed that the primary route of anastrozole elimination in the majority of models could be renal, thus implicating the drug-transporters. The rationale underlying the theories that anastrozole pharmacokinetics and bioavailability are influenced by modulation of transporters and potential variations in volume of distribution has already been discussed in *Section 4.3.2.2*. However, firm conclusions cannot be formed without a systematic analysis of drug transporter activity with respect to anastrozole. However, the proposed metabolic pathway of anastrozole **(Figure 4.1.2)** also implicates the UGTs in pharmacokinetic regulation of this drug, and in particular with bioavailability. Numerous human and mouse UGTs are regulated by PXR and CAR (Chen et al., 2003; Xie et al., 2003; Zhou et al., 2005; Buckley and Klaassen, 2009a; Li et al., 2009b; Yueh et al., 2011; Xu et al., 2012). Frequently this regulation is positive, yielding an increase in UGT expression. However, a recent report by Yueh *et al*. (2011) has demonstrated negative regulation of human UGT2B7, caused by CAR-mediated disruption of HNF-4α binding to promoter elements. This could be of particular relevance to those models demonstrating reduced anastrozole clearance as a result of the UGT-mediated metabolic pathways described by Kamdem *et al.* (2010)(**Figure 4.1.2)**. Another important feature of mouse UGT expression is that several isoforms demonstrate sexually dimorphic expression profiles (Buckley and Klaassen, 2009b), and thus could partially explain the gender-differential pharmacokinetics previously described.

Unfortunately, the UGT catalysed metabolism of anastrozole in mice has not been characterised with respect to specific isoforms, and consequently all hypotheses are based on human metabolic data. Although certain orthologues have been identified in mice, the significant divergence between the human and murine UGT systems prevents direct translation of metabolic data between species. For instance, the murine orthologue of the human *UGT1A4* gene,

ugt1a4, is a non-functional pseudogene (Buckley and Klaassen, 2007; Shiratani et al., 2008). In addition, no orthologue of the *UGT2B7* gene has been identified in mice, although it is clear that murine UGTs are capable of metabolising UGT2B7 catalysed reactions, such as morphine-O-glucuronidation (Buckley and Klaassen, 2007; Shiratani et al., 2008). A systematic analysis of anastrozole metabolism by UGTs in mice is essential to assess whether these enzymes have a meaningful role in anastrozole metabolism and to identify which isoforms are implicated. This analysis must also include tissue specific expresion/activity to assess the potential role of the UGT system in first-pass metabolism, and thus bioavailability.

4.4.3.3 ANASTROZOLE PHARMACOKINETICS DEMONSTRATE TEMPORAL VARIATION AND GENDER DIMORPHISM

Anastrozole pharmacokinetics demonstrate temporal variation in the majority of models, with this variation displaying both gender- and species-dimorphism **(Figures 4.3.13-4.3.16, Tables 4.3.3 & 4.3.4)**. Temporal variation in pharmacokinetics indicates a regulatory response which modulates the proteins associated with anastrozole disposition. This data set therefore indicates a regulatory interaction in the wild-type, PXR KO/HuCAR, mPXR/HuCAR, HuPXR/CAR KO and HuPXR/mCAR models. In the wild-type model, an increase in clearance of approximately 50% is consistently observed between the 1 and 3 day treatment cohorts in both sexes, and is associated with reduced drug exposure and $t_{½}$. However, there is no temporal variation in PK parameters in the PXR/CAR DHu model in either sex. This provides further evidence that anastrozole interacts with PXR/CAR in a species specific manner. Interestingly, temporal variation is not recorded in the mPXR/CAR KO or CAR KO/mPXR models, indicating that synergistic functioning between murine PXR/CAR is required to induce variation in the wild-type.

Data also indicates that synergism between the murine and human receptors can induce temporal variation. However, whereas increased clearance is observed in the mPXR/HuCAR model in both sexes, synergism between hPXR

and mCAR induces a temporal response in males only. Also, although no temporal variation is observed in the PXR/CAR DHu model, it is recorded in both the PXR KO/HuCAR and HuPXR/CAR KO models. However, the direction of change is dependent on gender, with clearance being enhanced in males between the two time points, but repressed in females. Interestingly, gender-specific induction in clearance is also observed in the PXR/CAR DKO model, with a female-specific induction in drug clearance. This clearly demonstrates that a non-PXR/CAR related regulatory mechanism is capable of modulating anastrozole disposition.

In summary, it is clear that PXR/CAR genotype has a significant influence on temporal variation in anastrozole pharmacokinetics, with a probable regulatory role in the wild-type, PXR KO/HuCAR, mPXR/HuCAR, HuPXR/CAR KO and HuPXR/mCAR models. Their ability to modulate pharmacokinetics in isolation suggests that hPXR and hCAR have a greater activity with respect to anastrozole than their murine counterparts, which require PXR to promote temporal change. However, synergy between hPXR and hCAR appears to be non-functional with respect to anastrozole-induced regulation of downstream proteins, thus demonstrating the importance of species-specific response to this analysis. Gender-specific response is also observed in the PXR KO/HuCAR, HuPXR/mCAR and HuPXR/mCAR models, although this data set is unable to provide any indication of the mechanism underlying the gender dimorphic effect. Further studies are therefore required to elucidate this phenomenon, potentially incorporating hormonal profiles, a full analysis of murine-specific anastrozole disposition and potential enzyme inhibition profiles. Data from the PXR/CAR DKO model is also suggestive of gender-specific regulation of genes involved in anastrozole disposition by a non-PXR/CAR-mediated regulatory system. Although variations in the PK parameters suggest the involvement of these receptors in the regulation of genes associated with anastrozole disposition, this alternative pathway needs to be further characterised before the role of PXR/CAR in the temporal variation of anastrozole pharmacokinetics can be fully assessed.

4.4.4 AROMATASE INHIBITORS INDUCE CYP2B6 IN A SPECIES- AND GENDER-SPECIFIC MANNER

It is clear from these observations that both anastrozole and letrozole induce the expression of the CYP2B6-lacZ reporter in this model **(Figures 4.3.17-4.3.19; Appendix A)**. Expression of the lacZ reporter is located in hepatic zone 2, in common with other data generated in our lab (Cameron & Chatham, unpublished). This is unusual because most P450 expression has been reported in the perivenous region (zone 3), although CYP2B6 expression has also been reported throughout zone 1 (Jungermann and Kietzmann, 1996; Wang and Tompkins, 2008; Colnot et al., 2011). Interestingly, the differential in magnitude observed mirrors that previously recorded for Cyp2b10, with anastrozole inducing a much stronger response than letrozole. This provides more evidence for the role of anastrozole as the stronger CAR activator, as previously discussed in *Section 4.3.1.3*.

Sexual dimorphism is also a notable feature of aromatase induced expression of CYP2B6, with stronger expression observed in the males than in the females in the mPXR/mCAR background CYP2B6-lacZ reporter line. This is a very unusual finding because the expression of CYP2B6, in common with that of Cyp2b10, has been reported to be greater in females than males (Lamba et al., 2003; Parkinson et al., 2004; Loryan et al., 2012), and thus it implies that this construct is not able to replicate gender-defined variations in expression. This could be impacted by modulations of oestrogen concentrations induced by aromatase inhibitor therapy, but given the primary role of growth hormone pulses in the control of gender-specific expression, this seems unlikely (Waxman et al., 1991; Jaffe et al., 2002; Dhir et al., 2006). Unfortunately, sexually dimorphic expression could not be assessed in the hPXR/hCAR CYP2B6-lacZ reporter line because of low mouse numbers. However, identifying the cause of this effect in both backgrounds could be imperative to the future utility of this model.

Finally, there is clear species-specificity in the regulation of CYP2B6 expression by PXR and CAR following letrozole treatment in males, with substantially lower expression observed in the model on the human background than on the murine. This presents two possibilities in terms of regulation. Firstly, the murine receptors have a greater affinity for the CYP2B6 promoter regions than their human counterparts, resulting in greater expression of the transgene. Secondly, that letrozole is a stronger activator of mPXR/mCAR than hPXR/hCAR with respect to CYP2B6. However, both scenarios could be caused by differential recruitment and interaction with other transcription factors and co-factors required for maximal CYP2B6 expression. A greater ability of the innate PXR/CAR system to interact with native co-factors would be consistent with the differential expression observed. Unfortunately, the low numbers of available mice have prevented full analysis of both aromatase inhibitors in both sexes, and thus reduced the evidence base for this observation. In order to further investigate this phenomenon, future experiments examining the CYP2B6-lacZ construct bred onto the full panel of PXR/CAR models would provide a greater insight into the species-specific regulation of CYP2B6 in these transgenic models.

In addition to reporter expression analysis, common blood biochemical markers were examined in these models **(Figure 4.3.20)**. The majority of markers showed no variation relative to vehicle control, although in part this could be ascribed to the significant inter-individual variation observed. However, of concern is the observation that creatinine concentration is significantly increased in the CYP2B6-lacZ model on the mPXR/mCAR background following letrozole treatment in males, and following both treatments in females. This indicates the possibility of kidney damage occurring in these models, a significant concern given that the clearance of both drugs is primarily renal in mice. Unfortunately kidney pathology was not examined and therefore the potential extent of any tissue damage cannot be assessed, although given the scale of this increase and the time-scale of the experiment this is unlikely to be significant.

In conclusion, it is clear that both aromatase inhibitors induce the expression of CYP2B6 in these models. Although significant analysis is still required to assess the cause of this species- and gender-specific induction, this could be an important clinical finding given the key role of CYP2B6 in the disposition of several chemotherapeutic agents, most notably cyclophosphamide (Wang and Tompkins, 2008). The induction of CYP2B6 in humans could potentially result in a greater risk of toxicity in subsequent cytotoxic treatment as a result of increased conversion of cyclophosphamide to its active moiety. The induction of CYP2B6 in humans in response to aromatase inhibitors should therefore be clarified with urgency to allow effective dose modulation to maintain clinical efficacy minimize overt toxicity in downstream treatment regimes.

4.5 Conclusions

This chapter demonstrates that both letrozole and anastrozole interact with PXR and CAR to modulate P450 expression and activity, as well as pharmacokinetics. Analysis has demonstrated that anastrozole exerts the stronger effect on P450 induction at the dose tested relative to letrozole, and that this effect is strongest in those models possessing the hCAR genotype, indicating the hCAR has a greater affinity for anastrozole than mCAR. The key role of CAR in the regulation of anastrozole-induced P450 expression is highlighted by the strong and species-differential induction of Cyp2b10 in all models possessing a CAR moiety. P450 modulation following letrozole treatment is more constrained than that observed following anastrozole treatment, and demonstrates notable gender-specificity, with Cyp2b10 induction seen only in females but the greatest Cyp3a11 induction observed in males. The former trait is mediated by CAR, being observed in females only in those models possessing a functional CAR protein, and has been attributed to the higher activity of Cyp2b10 and CAR previously reported in females (Jarukamjorn et al., 2002; Lamba et al., 2003; Petrick and Klaassen, 2007; Hernandez et al., 2009b). The latter trait has been associated with the activation of PXR, and particularly mPXR which shows the greatest activity following letrozole treatment regardless of CAR genotype, in contrast to hPXR,

which is unable to induce Cyp3a11 in the presence of CAR. Both nuclear receptors are therefore implicated in the induction of P450s following treatment with these drugs, although whether this is through direct or indirect interaction cannot be established from this dataset.

An unexpected finding was that the primary elimination route of both drugs in mice is potentially renal, and thus hepatic P450 metabolism could have a minimal impact on drug pharmacokinetics. This was corroborated by the lack of correlation between pharmacokinetic and protein expression trends. However, although P450 metabolism may have little role in the control of aromatase inhibitor pharmacokinetics in mice, it is evident that PXR and CAR are involved in the pharmacokinetic regulation of both drugs, although neither are obligate, and also that both receptors interact in a species-specific manner. This was best demonstrated by pharmacokinetic data obtained following letrozole treatment, in which the PXR/CAR DHu models demonstrated significantly faster drug elimination than wild-type, indicating that the human receptors have a greater activity with respect to letrozole than their murine orthologues. It was further demonstrated that hPXR promotes faster drug elimination in combination with CAR, mPXR represses innate CAR activity. CAR promotes faster drug elimination regardless of species, but this activity is modulated by PXR. Interestingly, PXR/CAR status is also a key determinant of temporal pharmacokinetic variation between the 1 day and 3 day cohorts, with PXR being found to drive variations between the two timepoints. However, there is also a gender differential effect observed, with mCAR also demonstrating activity with respect to temporal variation in females. These data therefore demonstrate the importance of assessing species- and gender-specific PXR and CAR activity, with the activity of both being differential.

Dissecting the role of PXR and CAR in anastrozole pharmacokinetics is more complex, in part because of the increased prevalence of sexual dimorphism, which is observed in the majority of genotypes. It is clear that murine and human PXR have a differential activity when functioning individually, with murine PXR enhancing anastrozole elimination whilst the human receptor represses elimination relative to wild-type. It is also evident that synergistic

functioning between PXR and CAR is an important element of anastrozole disposition, although there is marked gender dimorphism in terms of the importance and effects of receptor synergy. Most notably, the temporal variation observed in the wild-type model is lost in the PXR/CAR DHu model, regardless of sex. However, the presence of gender-dimorphic temporal variation in the species-hybrid and PXR/CAR DKO models makes it both impossible to dissect further receptor specific effects, and indicates the presence of a non-PXR/CAR-mediated regulatory mechanism which requires characterisation before the role of PXR and CAR in anastrozole-induced temporal mediation can be fully assessed.

Although hepatic P450 expression, and particularly that of Cyp3a11, may have a role in anastrozole metabolism, it appears to be of less relevance to letrozole metabolism. Other potential mediators of these effects are the drug-transporters, and, in the case of anastrozole, the UGTs, all of which have previously been associated with PXR/CAR-mediated regulation. These entities mediate drug influx to and efflux from the system and thus can significantly affect both absorption and excretion. However, this study does not have sufficient information to enable the role of these proteins in drug disposition to be fully analysed, and literature data regarding the elimination of letrozole and anastrozole in mice does not exist. Among the experiments required to address this problem are a systematic *in vitro* assessment of transporter interactions and UGT metabolism, primarily in the enterocytes, liver and kidneys. In order to address the role of gender-differential metabolism, the impact of AI therapy on oestrogen levels should also be assessed, as oestradiol and oestrone have both been shown to regulate PXR and CAR activity (Kawamoto et al., 2000; Mäkinen et al., 2003; Mnif et al., 2007; di Masi et al., 2009; Min, 2010; Dau et al., 2012; Koh et al., 2012). Without this basic data, the role of PXR and CAR in regulation of drug pharmacokinetics, and specifically the latter hypothesis, cannot be addressed. Also of importance is to increase the number of subjects enrolled in these studies, to enable more subtle trends to be dissected.

One weakness of this study is that our methodology only analyses pharmacokinetics from a loss of parent drug perspective, instead of analysing

both parental loss and generation of metabolites. This approach is therefore unable to specifically dissect disruptions in drug absorption, metabolism, and excretion relying instead on Western blot and activity data to provide support for any conclusions. However, a stronger approach would be to analyse both parent drug and its metabolites simultaneously to give more specific data regarding variations in pharmacokinetics, using a method such as has been recently described for letrozole in which letrozole and its two metabolites are simultaneously analysed by LC-MS/MS (Precht et al., 2012). At present no comparable method is available for the simultaneous analysis of anastrozole and its metabolites, most likely as a result of the comparative complexity of its metabolism. This methodology provides data to enable the impact of variations in P450-mediated metabolism to be directly related to pharmacokinetic effects, to detect any changes in UGT-mediated glucuronidation, as well as to help identify any non-metabolic processes affecting pharmacokinetics, such as transporter interactions that affect the absorption or excretion of the drug. To provide fuller supporting data for this technique, it is essential that a full *in vitro* analysis of drug metabolism in mice is performed to characterise the P450s, UGTs and drug transporters involved in the metabolism of these drugs.

Although this model panel has previously been used to assess species-specific regulatory crosstalk following phenobarbital treatment (Scheer et al., 2008), this is the first study to describe species-dependent variations in cross-talk in response to drug treatment. It is also the first study using these models to demonstrate the influence of gender-specificity on the regulation of P450-mediated metabolism. However, this study also highlights the importance of prior consideration of the role of regulatory cross-talk between nuclear receptors when using transgenic models in which a native gene has been replaced with one from another species. In both protein assays and pharmacokinetic analysis, notable disruptions have been identified in the two species-hybrid models (mPXR/HuCAR and HuPXR/mCAR) relative both to wild type and other related genotypes. It is highly probable that these variations are as a result of aberrant cross-talk between the two receptors, yielding pharmacokinetics and protein expression that is non-physiological. Although

this effect is limited in this study by the use of a full panel of PXR/CAR combinations, this is of particular concern given the widespread use of the single humanized models as a sole methodology, because without the full complement of genotype combinations, or corroborative evidence from an alternative model, aberrant receptor interactions cannot be identified. This could result in false conclusions being assigned to the non-native gene when the observation is actually driven by non-native crosstalk. This highlights the importance of selecting a methodology which can circumvent this issue, without which conclusions derived from the single-humanized models regarding regulatory control of xenobiotic metabolism must be treated with caution. This also highlights the importance of developing more complex models in which multiple genes are humanized, because it decreases the risk of aberrant crosstalk causing flawed data. However, without replacing all genes relating to drug metabolism and its control, aberrant crosstalk will always be a potential pitfall of using transgenic models, and therefore data should ideally be compared against that from a more clinically relevant model.

One final point of interest is the identification of a potential source of drug-drug interaction not previously described for these drugs, namely the potential induction of CYP2B6 by both aromatase inhibitors. Although this appears to be lower in the reporter line on a human PXR/CAR background, it is still identifiable. The lack of mouse numbers for the human background has limited the utility of this analysis. However, as a result of the role of CYP2B6 in drug disposition for many downstream chemotherapeutic agents, it is a finding that should be thoroughly investigated in more clinically relevant systems, such as primary human hepatocytes, liver slices, or ideally clinical trials, in which a probe substrate for CYP2B6, such as bupropion, could be employed to assess *in vivo* enzyme kinetics. Although this is a promising finding, it is important to note that these models require greater characterisation with respect to CYP2B6 regulation, with expression of the transgene being identified in an unusual location and with the reversal of expected sexually dimorphic induction patterns. Data generated should therefore be treated with caution.

Overall, this chapter has demonstrated that crosstalk between PXR and CAR is implicated in the regulation of letrozole and anastrozole pharmacokinetics. However, given that elimination of these drugs in mice is primarily renal, there is a limited relationship between P450 induction and pharmacokinetics, although the former is still of interest to the assessment of potential drug-drug interactions. The primary regulator of pharmacokinetics in this case is likely to be the drug transporters, and possibly the UGTs in the case of anastrozole. Significant research is still required to assess the involvement of these proteins, and their effects on the formation of metabolites. However, of particular importance is the demonstration that care must be taken when assigning conclusions to data from single humanized models, without first considering the role of aberrant crosstalk.

Study data therefore suggests that the null hypothesis for this study ("AIs interact with PXR/CAR in a species- and gender-specific manner to promote drug metabolism via the P450 enzyme system") is false, as a result of the potentially limited role of P450-mediated metabolism in AI disposition. However, further analysis of the disposition of these drugs is required to confirm this.

CHAPTER 5

A SUMMARY OF CONCLUSIONS

& FUTURE DIRECTIONS

5.1 AROMATASE INHIBITOR PHARMACOKINETICS ARE DEPENDENT ON PXR/CAR STATUS, BUT NOT P450-MEDIATED METABOLISM IN MICE

Nuclear receptor activation, and particularly that of PXR and CAR, is becoming increasingly recognised as a key determinant in the development of DDIs. Various methods are available to investigate the activation of these receptors in response to drug challenge, including reporter gene assays, primary human hepatocytes and certain simple transgenic mouse models. However, these models lack the sophistication to effectively assess inter-receptor cross-talk, a key regulatory mechanism in the control of drug metabolism. By using the novel panel of transgenic mouse models (Scheer et al., 2008; Ross et al., 2010; Scheer et al., 2010), this study was designed to specifically dissect the role of cross-talk between PXR and CAR in the metabolism and pharmacokinetics of commonly available pharmaceuticals. This approach also allowed species-differential activation of these nuclear receptors, as well as the potential impact of aberrant cross-talk between receptors of two species, to be analysed.

In the first phase of the study (*Chapter 3)*, the aromatase inhibitors, letrozole and anastrozole, were identified as potential activators of PXR and CAR, on the basis of their induction of the prototypical target genes, Cyp3a11 and Cyp2b10, and thus were selected for further study. In these early experiments, sexually dimorphic P450 activity was also observed, potentially suggesting sex-specific changes in PXR/CAR activity. Gefitinib and cyclophosphamide were also implicated as ligands for both nuclear receptors, but demonstrated a lower response level than letrozole, and thus were not selected. However, with respect to cyclophosphamide, these data agree with a recent study which identifies cyclophosphamide as an activator of both PXR and CAR (Wang et al., 2011).

Interaction with PXR and CAR is a key modulator of P450 expression and activity, as well as drug pharmacokinetics (*Chapter 4*). The activity of PXR/CAR was species-specific with respect to AI pharmacokinetics. Letrozole has higher

activity with respect to human PXR/CAR than murine, resulting in significantly faster drug elimination. Species-specificity in letrozole response is predominantly driven by PXR modulation of CAR activity, with CAR being essential for higher drug metabolism, irrespective of species. PXR has also been identified as the key mediator of temporal changes in pharmacokinetics, although mCAR also demonstrates activity in the female cohort only. Anastrozole pharmacokinetics also demonstrate species-specificity with respect to PXR, although in this case mPXR demonstrates the higher activity. However, gender-dimorphic pharmacokinetics observed in the majority of genotypes complicates the identification of genotypic influences. Limited conclusions could therefore be drawn regarding receptor interactions, although synergistic functioning between PXR/CAR remains an important regulatory factor. There is also evidence of PXR/CAR dependency in the control of temporal variation in anastrozole pharmacokinetics. However, although AI pharmacokinetics are modulated by PXR/CAR genotype, neither PXR/CAR are obligate for drug elimination, therefore suggesting a role for an alternative regulatory pathway. The data also illustrate the importance of considering potential aberrant cross-talk between non-species linked nuclear receptors to avoid false conclusions derived from single humanized models. Failure to consider the potential for non-native cross-talk and to plan methodology to limit its potential impact could impact the relevance of data generated in these models.

Contrary to published data derived from humans and rats, drug elimination in mice appears to rely on renal, as opposed to hepatic excretion, although further studies are required to confirm this hypothesis. If proven, it is most likely that the PXR/CAR mediated modulations in AI pharmacokinetics are as a result of modulation of drug transporter or UGT activity. However, there is still notable induction of P450s with respect to many genotypes, in particular Cyp3a11 and Cyp2b10, a finding which could have significant ramifications for combination therapy. Of particular note is the induction of Cyp2b10 in all models expressing CAR, which has not been reported in previous analyses of P450 induction following AI treatment. Species-specific response demonstrates higher activity in hPXR models than mPXR, with the latter requiring interaction with CAR. The

data also demonstrate gender-specific regulation of this gene following both treatments, with expression being higher in females than males, in agreement with previous studies indicating a female-specific increase in CAR activity (Jarukamjorn et al., 2002; Lamba et al., 2003; Petrick and Klaassen, 2007; Hernandez et al., 2009b). It also suggests that anastrozole is a stronger inducer of CAR than letrozole. Induction of the CYP2B6-LacZ reporter was also observed in all samples following drug treatment, although response is strongest in models under the control of murine PXR/CAR, as well as in males. This observation therefore has potential importance in human pharmacokinetic studies. Further characterization of these models, combined with analysis of CYP2B6 induction in a relevant human model, such as the HepaRG cell line (see below), is required to confirm this finding. However, the identification of previously unreported induction of Cyp2b10/CYP2B6 could have significant therapeutic implications for those drugs that rely on these enzymes for their metabolism. A key example is cyclophosphamide, a drug widely used in the treatment of breast cancer, for which CYP2B6 is essential for the conversion of the pro-drug to the active moiety, and therefore this effect should be further investigated as a matter of urgency.

In summary, this study has identified a number of drugs with the potential to interact with PXR and CAR. It has further demonstrated that PXR and CAR are activated by the AIs, and that this activation is reflected in variations in pharmacokinetics. However, in contrast to published evidence, this is unlikely to be a result of P450-mediated induction, with pharmacokinetics suggesting possible renal clearance and thus implicating the drug transporters and UGTs. This also indicates that although evidence suggests interaction with PXR and CAR, a mouse model is not appropriate for the full analysis of the metabolism of these drugs, and therefore the findings of this study should be progressed to a more relevant model to provide corroboration. It is essential to consider cross-talk between these receptors, with species-specific effects having been observed in both pharmacokinetics and protein expression, in addition to aberrant pharmacokinetics and protein activity in non-species linked receptor models with respect to AIs. Gender-specificity is also evident in the response to

drug treatment, although further research needs to be performed to identify if the effect is mediated by direct binding of AIs to the nuclear receptors, or whether it is mediated indirectly through another pathway. Of particular interest is the oestrogen receptor pathway, with 17β-oestradiol and oestrone having both shown activity with respect to PXR and CAR, and with which system the AIs interact (Kawamoto et al., 2000; Mäkinen et al., 2003; Mnif et al., 2007; di Masi et al., 2009; Min, 2010; Dau et al., 2012; Koh et al., 2012). In conclusion, although the PXR/CAR model panel was not appropriate for the analysis of AI drug metabolism, they have demonstrated their efficacy for the assessment of regulatory cross-talk and should be considered a useful tool for future studies.

5.2 Future directions

5.2.1 AI study: Assessing drug-transporter expression and activity

In order to provide data to support the hypotheses suggested, a systematic analysis of AI metabolism, encompassing P450s, UGTs and drug transporters in these subjects is required. Studies should include recombinant enzymes/transporters, in addition to relevant cell fractions derived from frozen liver and intestinal fractions. Unfortunately, relevant kidney samples are not available, and therefore differential modulation of renal transporter/UGT expression and activity cannot be examined without repeating the study in a new cohort of mice. These studies should also include experiments to specifically analyse renal excretion, and include pharmacokinetic studies examining the creation of metabolites, in order to provide a robust basis for conclusions regarding primary route of elimination in mice. However, given that the metabolism of the AIs in mice and humans appears to be very different, any further use of mice for this purpose is unlikely to be ethical and therefore careful consideration should be given as to the ethics of performing this study.

5.2.2 CHARACTERISATION OF AI INTERACTIONS WITH PXR/CAR IN *IN VITRO* MODELS

As discussed above, mouse models are clearly not suitable for the assessment of the effects of PXR/CAR activation on AI pharmacokinetics as a result of species differential metabolism. However, the PXR/CAR related modulations in pharmacokinetics identified in mice does suggest some form of interaction with these nuclear receptors, and thus requires further analysis in a more relevant model.

One such model is that of the HepaRG cell line. First described in 2002, this cell line was derived from hepatocarcinoma isolated from a female patient, and differentiates into a co-culture of biliary and hepatocyte-like cells (Gripon et al., 2002; Kanebratt and Andersson, 2008a; Kanebratt and Andersson, 2008b). It is unusual for an immortal cell line in that it stably expresses many elements of the drug metabolism pathways following differentiation, including P450s, Phase II enzymes, drug transporters, and the nuclear receptors, PXR and CAR, at levels close to those identified in hepatocytes (Gripon et al., 2002; Aninat et al., 2006; Le Vee et al., 2006; Anthérieu et al., 2010; Anthérieu et al., 2012). In addition, once differentiated, this cell line exhibits limited changes in gene expression profile for four weeks, provided they are not DMSO deprived (Jossé et al., 2008; Anthérieu et al., 2010; Anthérieu et al., 2012). This allows the analysis of drug metabolism in an immortal cell line, thus reducing study variability and also removing three of the major problems associated with studies in PHHs, requiring no ethical approval, having no issues with supply and being stable for prolonged, although not indefinite, periods of time. Monolayer cultures have therefore become a common model used for the assessment of drug toxicity and metabolism (Guillouzo et al., 2007; Kanebratt and Andersson, 2008a; Kanebratt and Andersson, 2008b; Kaneko et al., 2009; Lambert et al., 2009a; Lambert et al., 2009b; McGinnity et al., 2009; Turpeinen et al., 2009; Andersson et al., 2012). Recently, the power of this model has been increased by the development of 3D culture bioreactors which demonstrate *in vivo* liver-like

morphology, and which also appears to extend the stability of this cell line with respect to drug metabolism (Darnell et al., 2011; Darnell et al., 2012; Leite et al., 2012; Nibourg et al., 2012a; Nibourg et al., 2012b; Nibourg et al., 2012c). Of particular interest is their potential use as a biocomponent in bioartificial liver reactors, with a recent study demonstrating their efficacy in this role, with survival time being increased by 50% in rats with acute liver failure (Hoekstra et al., 2011; Nibourg et al., 2012a; Nibourg et al., 2012b). This model therefore has significant potential for use both in toxicity testing, as well as in the support of those with severe liver suppression.

With respect to this study, the HepaRG model could be used to provide further clarification of the role of PXR and CAR in the regulation of AI metabolism. The stability of expression demonstrated by this cell line means that extended drug treatments can be used, and thus delayed metabolic reactions to drug challenge can be analysed. *In situ* pharmacokinetic studies could also be performed at several points over an extended period, allowing temporal variations to be analysed. Assays involving prototypical probe substrates for elements of the drug elimination cascade could also be employed to give real-time, *in situ* analysis of changes in protein activity modulation in these cultures (Feidt et al., 2010; Videau et al., 2010; Prot et al., 2011; Rhodes et al., 2011; Kozakai et al., 2012; Liu et al., 2012). One further peculiarity of this cell line is its ability to be transfected, something that is traditionally challenging in a hepatocyte cell line (Laurent et al., 2010). This presents the possibility of introducing siRNA targeted against PXR/CAR to enable the role of cross-talk between these receptors in the regulation of AI pharmacokinetics to be assessed in a relevant hepatocyte-like model. With correct methodology selection, experiments in this model could therefore provide significant evidence to corroborate or disprove those observations generated in the mouse panel with respect to AI interactions with PXR/CAR.

In order to provide a more mechanistic assessment of AI interactions with PXR/CAR, reporter gene assays can be employed. To facilitate this, I have adapted the mPXR and hPXR luciferase reporter assays kindly donated by Luisella A. Vignati (Perlmann and Jansson, 1995; Bertilsson et al., 1998;

Blumberg et al., 1998; Vignati et al., 2004), to enable the activation of mCAR and hCAR by direct ligand binding to be assessed. The reporter consists of the LBD of mCAR/hCAR linked to the DNA-binding domain of GAL-4. Once activated by CAR ligand binding, the GAL-4 DNA-binding motif binds to the GAL-4 recognition sequence linked to luciferase on a second plasmid, thus inducing luciferase induction. The pCMX Gal4-mCAR-LBD model has been fully validated and previously published (Finn et al., 2009). The pCMX Gal4-hCAR-LBD is still awaiting full validation. Once completed, this panel of assays will allow the transcriptional activity of PXR/CAR in response to direct ligand induction to be assessed, and thus could provide useful data regarding species-dependent interactions of the AIs with these receptors. However, one limitation of these models is that they are unable to analyse induction caused by indirect activation, and therefore data should be combined with other experimental approaches, such as the HepaRG approach described above.

5.2.3 Developing the study methodology

Although the mouse model panel was not suitable for the assessment of AI pharmacokinetic modulation, the variations observed clearly demonstrate its utility for PXR/CAR regulatory cross-talk studies investigating other drugs. It could therefore be of use in the analysis of those other drugs in *Chapter 3* identified as potential modulators of PXR/CAR activity. However, one aspect lacking from the current methodology is a link from pharmacokinetic modulation to changes in tumour growth. Ideally, this panel would be bred onto an immuno-deficient background, such as SCID, or immunocompromised through neonatal thymectomy + gamma irradiation, to enable xenograft studies, and thus provide a more clinically relevant output. However, the former is a costly and time-consuming process, whilst the latter is complex and requires ethical approval. An alternative methodology to avoid these problems is to use a syngeneic tumour model, in which mice are implanted with cells from a tumour raised in a relevant murine background. Although the clinical relevance is likely to be reduced by this approach, it is able to give important information

regarding variations in drug efficacy in a physiological system which can then be translated to other models.

As a result of the tumour-resistant properties of the C57BL/6J background, there are fewer models available for use in this panel than those derived on alternative backgrounds, such as BALB/C. However, among such models, one that has been well characterised is the E0771 cell line (Sirotnak et al., 1993; Ewens et al., 2005; Ewens et al., 2006; Gu et al., 2009; Stagg et al., 2010; Gu et al., 2011). E0771 is an aggressive medullary breast adenocarcinoma derived from a C57BL/6J background, and thus can be implanted into the PXR/CAR mouse panel. When injected I.V. into the tail vein, extensive metastatic lung cancer is engendered, resulting in death within 12-15 days (Sirotnak et al., 1993). However, when grown more conventionally, by sub-cutaneous injection near the 4th mammary fat pad, the doubling rate for these tumours is approximately 7 days, with all mice possessing tumours having been euthanized by 50 days post-injection (Ewens et al., 2005). There is also a low failure rate following transplantation, with 97% of all subjects developing identifiable tumours. These therefore demonstrate potential utility for studies investigating drugs commonly used in the treatment of breast cancer.

A small-scale initial characterisation experiment was therefore performed in wild-type C57BL/6J and PXR/CAR DHu mice, to assess growth kinetics in these two models, although the cells were injected sub-cutaneously into the flanks of each mouse. This initial study demonstrated both that growth kinetics were comparable to those published by Ewens *et al.* (2005), and also that there was no difference in growth kinetics between the two genotypes **(Appendix B)**. Tumours developed in 9/10 of subjects following injection, 5 of which demonstrate muscle attachment (only 1 with possible invasion), 1 being attached to skin and 2 showing no attachment. In contrast to the study by Ewens *et al*. (2005), no distant metastases were identified on visual inspection. However, it is likely that this is as a result of the more conservative requirements regarding maximum tumour size employed in this study, requiring euthanasia when the tumour reaches a GMD of 12.5mm^2 instead of the larger measurement of 20mm in any dimension used by Ewens *et al*. (2005).

This reduces the risk of invasion, with only one subject showing any potential muscle-layer invasion in this study, whereas 76% of subjects demonstrated invasion of peritoneal muscle in the Ewens *et al*. (2005) study. These data suggest that the E0771 model can be effectively used in the transgenic PXR/CAR mouse models to provide a direct readout of therapeutic efficacy. However, prior to initiating such studies, a larger scale characterisation study is required to fully assess growth kinetics in each of the different genotypes, as a result of potential differential modulation of inflammatory processes associated with the individual PXR/CAR transgenics which could interfere with published growth kinetics.

CHAPTER 6
REFERENCES

6.1 REFERENCES

Andersson TB, Kanebratt KP and Kenna JG (2012) The HepaRG cell line: A unique in vitro tool for understanding drug metabolism and toxicology in human. *Expert Opinion on Drug Metabolism and Toxicology* **8:**909-920.

Aninat C, Piton A, Glaise D, Le Charpentier T, Langouët S, Morel F, Guguen-Guillouzo C and Guillouzo A (2006) Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metabolism and Disposition* **34:**75-83.

Anthérieu S, Chesné C, Li R, Camus S, Lahoz A, Picazo L, Turpeinen M, Tolonen A, Uusitalo J, Guguen-Guillouzo C and Guillouzo A (2010) Stable expression, activity, and inducibility of cytochromes P450 in differentiated HepaRG cells. *Drug Metabolism and Disposition* **38:**516-525.

Anthérieu S, Chesné C, Li R, Guguen-Guillouzo C and Guillouzo A (2012) Optimization of the HepaRG cell model for drug metabolism and toxicity studies. *Toxicology in Vitro*.

Azuma K, Urano T, Watabe T, Ouchi Y and Inoue S (2011) PROX1 suppresses vitamin K-induced transcriptional activity of steroid and xenobiotic receptor. *Genes to Cells* **16:**1063-1070.

Bachmann KA (2006) Inhibition constants, inhibitor concentrations and the prediction of inhibitory drug drug interactions: Pitfalls, progress and promise. *Current Drug Metabolism* **7:**1-14.

Baes M, Gulick T, Choi HS, Martinolu MG, Simha D and Moore DD (1994) A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Molecular and Cellular Biology* **14:**1544-1552.

Bao T, Blackford AL and Stearns V (2012) Effect of simvastatin on the pharmacokinetics of anastrozole. *Breast Cancer Research and Treatment* **131:**709-711.

Barwick JL, Quattrochi LC, Mills AS, Potenza C, Tukey RH and Guzelian PS (1996) Trans-species gene transfer for analysis of glucocorticoid-inducible transcriptional activation of transiently expressed human CYP3A4 and rabbit CYP3A6 in primary cultures of adult rat and rabbit hepatocytes. *Molecular Pharmacology* **50:**10-16.

Bebawy M and Chetty M (2009) Gender differences in P-glycoprotein expression and function: Effects on drug disposition and outcome. *Current Drug Metabolism* **10:**322-328.

Beilke LD, Aleksunes LM, Holland RD, Besselsen DG, Beger RD, Klaassen CD and Cherrington NJ (2009) Constitutive androstane receptor-mediated changes in bile acid composition contributes to hepatoprotection from lithocholic acid-Induced liver injury in mice. *Drug Metabolism and Disposition* **37:**1035-1045.

Ben-Haroush A, Farhi J, Ben-Aharon I, Sapir O, Pinkas H and Fisch B (2011) High yield of oocytes without an increase in circulating estradiol levels in breast cancer patients treated with follicle-stimulating hormone and aromatase inhibitor in standard gonadotropin-releasing hormone analogue protocols. *Israel Medical Association Journal* **13:**752-755.

Benet M, Lahoz A, Guzman C, Castell JV and Jover R (2010a) CCAAT/enhancer-binding protein α (C/EBPα) and hepatocyte nuclear factor 4α (HNF4α) synergistically cooperate with constitutive androstane receptor (CAR) to transactivate the human cytochrome P450 2B6 (CYP2B6) gene: Application to the development of a metabolically competent human hepatic cell model. *Journal of Biological Chemistry* **doi: 10.1074/jbc.M110.118364**.

Benet M, Lahoz A, Guzmán C, Castell JV and Jover R (2010b) CCAAT/enhancer-binding protein α (C/EBPα) and hepatocyte nuclear factor 4α (HNF4α) synergistically cooperate with constitutive androstane receptor to transactivate the human cytochrome P450 2B6 (CYP2B6) gene: Application to the development of a metabolically competent human hepatic cell model. *Journal of Biological Chemistry* **285:**28457-28471.

Benowitz NL, Lessov-Schlaggar CN, Swan GE and Jacob Iii P (2006) Female sex and oral contraceptive use accelerate nicotine metabolism. *Clinical Pharmacology and Therapeutics* **79:**480-488.

Bertilsson G, Heidrich J, Svensson K, Åsman M, Jendeberg L, Sydow-Bäckman M, Ohlsson R, Postlind H, Blomquist P and Berkenstam A (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proceedings of the National Academy of Sciences of the United States of America* **95:**12208-12213.

Biegon A, Kim SW, Alexoff DL, Jayne M, Carter P, Hubbard B, King P, Logan J, Muench L, Pareto D, Schlyer D, Shea C, Telang F, Wang GJ, Xu Y and Fowler JS (2010) Unique distribution of aromatase in the human brain: In vivo studies with PET and [N-methyl- 11C]vorozole. *Synapse* **64:**801-807.

Bilotas M, Meresman G, Stella I, Sueldo C and Barañao RI (2010) Effect of aromatase inhibitors on ectopic endometrial growth and peritoneal environment in a mouse model of endometriosis. *Fertility and Sterility* **93:**2513-2518.

Biswas A, Pasquel D, Tyagi RK and Mani S (2011) Acetylation of pregnane X receptor protein determines selective function independent of ligand activation. *Biochemical and Biophysical Research Communications* **406:**371-376.

Blättler SM, Rencurel F, Kaufmann MR and Meyer UA (2007) In the regulation of cytochrome P450 genes, phenobarbital targets LKB1 for necessary activation of AMP-activated protein kinase. *Proceedings of the National Academy of Sciences of the United States of America* **104:**1045-1050.

Blomster HA, Imanishi SY, Siimes J, Kastu J, Morrice NA, Eriksson JE and Sistonen L (2010) In vivo identification of sumoylation sites by a signature tag and cysteine-targeted affinity purification. *Journal of Biological Chemistry* **285:**19324-19329.

Blumberg B, Sabbagh Jr W, Juguilon H, Bolado Jr J, Van Meter CM, Ong ES and Evans RM (1998) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes and Development* **12:**3195-3205.

Bock KW (2010) Functions and transcriptional regulation of adult human hepatic UDP-glucuronosyl-transferases (UGTs): Mechanisms responsible for interindividual variation of UGT levels. *Biochemical Pharmacology* **80:**771-777.

Bock KW (2011) From differential induction of UDP-glucuronosyltransferases in rat liver to characterization of responsible ligand-activated transcription factors, and their multilevel crosstalk in humans. *Biochemical Pharmacology* **82:**9-16.

Bombail V, Taylor K, Gibson GG and Plant N (2004) Role of Sp1, C/EBPα, HNF3, and PXR in the basal- and xenobiotic-mediated regulation of the CYP3A4 gene. *Drug Metabolism and Disposition* **32:**525-535.

Borrelli F and Izzo AA (2009) Herb-drug interactions with St John's Wort (hypericum perforatum): An update on clinical observations. *AAPS Journal* **11:**710-727.

Boverhof DR, Chamberlain MP, Elcombe CR, Gonzalez FJ, Heflich RH, Hernández LG, Jacobs AC, Jacobson-Kram D, Luijten M, Maggi A, Manjanatha MG, van Benthem J and Gollapudi BB (2011) Transgenic animal models in toxicology: Historical perspectives and future outlook. *Toxicological Sciences* **121:**207-233.

Brandon EFA, Raap CD, Meijerman I, Beijnen JH and Schellens JHM (2003) An update on in vitro test methods in human hepatic drug biotransformation research: Pros and cons. *Toxicology and Applied Pharmacology* **189:**233-246.

Buajordet I, Ebbesen J, Erikssen J, BrØrs O and Hilberg T (2001) Fatal adverse drug events: The paradox of drug treatment. *Journal of Internal Medicine* **250:**327-341.

Buckley DB and Klaassen CD (2007) Tissue- and gender-specific mRNA expression of UDP-glucuronosyltransferases (UGTs) in mice. *Drug Metabolism and Disposition* **35:**121-127.

Buckley DB and Klaassen CD (2009a) Induction of mouse UDP-glucuronosyltransferase mRNA expression in liver and intestine by activators of aryl-hydrocarbon receptor, constitutive androstane receptor, pregnane X

receptor, peroxisome proliferator-activated receptorα, and nuclear factor erythroid 2-related factor 2. *Drug Metabolism and Disposition* **37:**847-856.

Buckley DB and Klaassen CD (2009b) Mechanism of gender-divergent UDP-glucuronosyltransferase mRNA expression in mouse liver and kidney. *Drug Metabolism and Disposition* **37:**834-840.

Bucşa C, Farcaş A, Cazacu I, Leucuta D, Achimas-Cadariu A, Mogosan C and Bojita M (2012) How many potential drug-drug interactions cause adverse drug reactions in hospitalized patients? *European Journal of Internal Medicine* ***In Press***.

Bulun SE, Chen D, Lu M, Zhao H, Cheng Y, Demura M, Yilmaz B, Martin R, Utsunomiya H, Thung S, Su E, Marsh E, Hakim A, Yin P, Ishikawa H, Amin S, Imir G, Gurates B, Attar E, Reierstad S, Innes J and Lin Z (2007) Aromatase excess in cancers of breast, endometrium and ovary. *Journal of Steroid Biochemistry and Molecular Biology* **106:**81-96.

Bulun SE and Simpson ER (2008) Aromatase expression in women's cancers. *Advances in Experimental Medicine and Biology* **630:**112-132.

Burk O, Arnold KA, Nussler AK, Schaeffeler E, Efimova E, Avery BA, Avery MA, Fromm MF and Eichelbaum M (2005) Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. *Molecular Pharmacology* **67:**1954-1965.

Buzdar AU, Jonat W, Howell A and Plourde PV (1997) ARIMIDEX: A potent and selective aromatase inhibitor for the treatment of advanced breast cancer. *Journal of Steroid Biochemistry and Molecular Biology* **61:**145-149.

Campbell SJ, Henderson CJ, Anthony DC, Davidson D, Clark AJ and Wolf CR (2005) The murine Cyp1a1 gene is expressed in a restricted spatial and temporal pattern during embryonic development. *Journal of Biological Chemistry* **280:**5828-5835.

Carlson RW, Theriault R, Schurman CM, Rivera E, Chung CT, Phan SC, Arun B, Dice K, Chiv VY, Green M and Valero V (2010) Phase II trial of anastrozole plus goserelin in the treatment of hormone receptor-positive, metastatic carcinoma of the breast in premenopausal women. *Journal of Clinical Oncology* **28:**3917-3921.

Cashman J, Wright J and Ring A (2010) The treatment of co-morbidities in older patients with metastatic cancer. *Supportive Care in Cancer* **18:**651-655.

Cavallini G, Beretta G and Biagiotti G (2011) Preliminary study of letrozole use for improving spermatogenesis in non-obstructive azoospermia patients with normal serum FSH. *Asian Journal of Andrology* **13:**895-897.

Chan GNY, Hoque MT, Cummins CL and Bendayan R (2011) Regulation of P-glycoprotein by orphan nuclear receptors in human brain microvessel endothelial cells. *Journal of Neurochemistry* **118:**163-175.

Chang TKH, Yu L, Maurel P and Waxman DJ (1997) Enhanced cyclophosphamide and ifosfamide activation in primary human hepatocyte cultures: Response to cytochrome P-450 inducers and autoinduction by oxazaphosphorines. *Cancer Research* **57:**1946-1954.

Charles KA, Rivory LP, Brown SL, Liddle C, Clarke SJ and Robertson GR (2006) Transcriptional repression of hepatic Cytochrome P450 3A4 gene in the presence of cancer. *Clinical Cancer Research* **12:**7492-7497.

Chen C, Staudinger JL and Klaassen CD (2003) Nuclear receptor, pregnane X receptor, is required for induction of UDP-glucuronosyltransferases in mouse liver by 16aÎ±-carbonitrile. *Drug Metabolism and Disposition* **31:**908-915.

Chen Y and Goldstein JA (2009) The transcriptional regulation of the human CYP2C genes. *Current Drug Metabolism* **10:**567-578.

Chen Y, Tang Y, Guo C, Wang J, Boral D and Nie D (2012) Nuclear receptors in the multidrug resistance through the regulation of drug-metabolizing enzymes and drug transporters. *Biochemical Pharmacology* **83:**1112-1126.

Chen Y, Tang Y, Robbins GT and Nie D (2010) Camptothecin attenuates cytochrome P450 3A4 induction by blocking the activation of human pregnane X receptor. *Journal of Pharmacology and Experimental Therapeutics* **334:**999-1008.

Cheng J, Ma X and Gonzalez FJ (2011) Pregnane X receptor- and CYP3A4-humanized mouse models and their applications. *British Journal of Pharmacology* **163:**461-468.

Cheung C and Gonzalez FJ (2008) Humanized mouse lines and their application for prediction of human drug metabolism and toxicological risk assessment. *Journal of Pharmacology and Experimental Therapeutics* **327:**288-299.

Cheung KL, Agrawal A, Folkerd E, Dowsett M, Robertson JFR and Winterbottom L (2010) Suppression of ovarian function in combination with an aromatase inhibitor as treatment for advanced breast cancer in pre-menopausal women. *European Journal of Cancer* **46:**2936-2942.

Chirgwin J, Sun Z, Smith I, Price KN, Thürlimann B, Ejlertsen B, Bonnefoi H, Regan MM, Goldhirsch A and Coates AS (2012) The advantage of letrozole over tamoxifen in the BIG 1-98 trial is consistent in younger postmenopausal women and in those with chemotherapy-induced menopause. *Breast Cancer Research and Treatment* **131:**295-306.

Choi HS, Chung M, Tzameli I, Simha D, Lee YK, Seol W and Moore DD (1997) Differential transactivation by two isoforms of the orphan nuclear hormone receptor CAR. *Journal of Biological Chemistry* **272:**23565-23571.

Chrencik JE, Orans J, Moore LB, Xue Y, Peng L, Collins JL, Wisely GB, Lambert MH, Kliewer SA and Redinbo MR (2005) Structural disorder in the complex of human pregnane X receptor and the macrolide antibiotic rifampicin. *Molecular Endocrinology* **19:**1125-1134.

Chu QSC, Cianfrocca ME, Goldstein LJ, Gale M, Murray N, Loftiss J, Arya N, Koch KM, Pandite L, Fleming RA, Paul E and Rowinsky EK (2008) A phase I and pharmacokinetic study of lapatinib in combination with letrozole in patients with advanced cancer. *Clinical Cancer Research* **14:**4484-4490.

Chumsri S, Howes T, Bao T, Sabnis G and Brodie A (2011) Aromatase, aromatase inhibitors, and breast cancer. *Journal of Steroid Biochemistry and Molecular Biology* **125:**13-22.

Coleman MD (2010) *Human Drug Metabolism: An Introduction*. Wiley.

Colleoni M, Giobbie-Hurder A, Regan MM, Thürlimann B, Mouridsen H, Mauriac L, Forbes JF, Paridaens R, Láng I, Smith I, Chirgwin J, Pienkowski T, Wardley A, Price KN, Gelber RD, Coates AS and Goldhirsch A (2011) Analyses adjusting for selective crossover show improved overall survival with adjuvant letrozole compared with tamoxifen in the BIG 1-98 study. *Journal of Clinical Oncology* **29:**1117-1124.

Collins JM (2001) Inter-species differences in drug properties. *Chemico-Biological Interactions* **134:**237-242.

Colnot S, Perret C and Monga SPS (2011) Liver Zonation, in: *Molecular Pathology of Liver Diseases*, pp 7-16, Springer US.

Colussi DM, Parisot CY and Lefèvre GY (1998) Plasma protein binding of letrozole, a new nonsteroidal aromatase enzyme inhibitor. *Journal of Clinical Pharmacology* **38:**727-735.

Crivellari D, Sun Z, Coates AS, Price KN, Thürlimann B, Mouridsen H, Mauriac L, Forbes JF, Paridaens RJ, Castiglione-Gertsch M, Gelber RD, Colleoni M, Láng I, Del Mastro L, Gladieff L, Rabaglio M, Smith IE, Chirgwin JH and Goldhirsch A (2008) Letrozole compared with tamoxifen for elderly patients with endocrine-responsive early breast cancer: The BIG 1-98 trial. *Journal of Clinical Oncology* **26:**1972-1979.

Cui X, Thomas A, Gerlach V, White RE, Morrison RA and Cheng KC (2008) Application and interpretation of hPXR screening data: Validation of reporter signal requirements for prediction of clinically relevant CYP3A4 inducers. *Biochemical Pharmacology* **76:**680-689.

Cuzick J, Sestak I, Baum M, Buzdar A, Howell A, Dowsett M and Forbes JF (2010) Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 10-year analysis of the ATAC trial. *The Lancet Oncology* **11:**1135-1141.

Damery S, Gratus C, Grieve R, Warmington S, Jones J, Routledge P, Greenfield S, Dowswell G, Sherriff J and Wilson S (2011) The use of herbal medicines by people with cancer: A cross-sectional survey. *British Journal of Cancer* **104:**927-933.

Darnell M, Schreiter T, Zeilinger K, Urbaniak T, Söderdahl T, Rossberg I, Dillnér B, Berg AL, Gerlach JC and Andersson TB (2011) Cytochrome P450-dependent metabolism in HepaRG cells cultured in a dynamic three-dimensional bioreactor. *Drug Metabolism and Disposition* **39:**1131-1138.

Darnell M, Ulvestad M, Ellis E, Weidolf L and Andersson TB (2012) In vitro evaluation of major in vivo drug metabolic pathways using primary human hepatocytes and HepaRG cells in suspension and a dynamic three-dimensional bioreactor system. *Journal of Pharmacology and Experimental Therapeutics* **343:**134-144.

Dau PT, Sakai H, Hirano M, Ishibashi H, Tanaka Y, Kameda K, Fujino T, Kim E-Y and Iwata H (2012) Quantitative Analysis of the Interaction of Constitutive Androstane Receptor with Chemicals and Steroid Receptor Coactivator 1 Using Surface Plasmon Resonance Biosensor Systems: A Case Study of the Baikal Seal (Pusa sibirica) and the Mouse. *Toxicological Sciences***:***In Press*.

Davies B and Morris T (1993) Physiological parameters in laboratory animals and humans. *Pharmaceutical Research* **10:**1093-1095.

Davies EC, Green CF, Taylor S, Williamson PR, Mottram DR and Pirmohamed M (2009) Adverse drug reactions in hospital in-patients: A prospective analysis of 3695 patient-episodes. *PLoS ONE* **4**.

de Ronde W and de Jong FH (2011) Aromatase inhibitors in men: Effects and therapeutic options. *Reproductive Biology and Endocrinology* **9**.

DeGraw JI, Colwell WT, Piper JR and Sirotnak FM (1993) Synthesis and antitumor activity of 10-propargyl-10-deazaaminopterin. *Journal of Medicinal Chemistry* **36:**2228-2231.

Dent SF, Gaspo R, Kissner M and Pritchard KI (2011) Aromatase inhibitor therapy: Toxicities and management strategies in the treatment of postmenopausal women with hormone-sensitive early breast cancer. *Breast Cancer Research and Treatment* **126:**295-310.

Desai PB, Nallani SC, Sane RS, Moore LB, Goodwin BJ, Buckley DJ and Buckley AR (2002) Induction of cytochrome P450 3A4 in primary human hepatocytes and activation of the human pregnane X receptor by tamoxifen and 4-hydroxytamoxifen. *Drug Metabolism and Disposition* **30:**608-612.

Desta Z, Kreutz Y, Nguyen AT, Li L, Skaar T, Kamdem LK, Henry NL, Hayes DF, Storniolo AM, Stearns V, Hoffmann E, Tyndale RF and Flockhart DA (2011) Plasma letrozole concentrations in postmenopausal women with breast cancer are associated with CYP2A6 genetic variants, body mass index, and age. *Clinical Pharmacology and Therapeutics* **90:**693-700.

Desta Z, Ward BA, Soukhova NV and Flockhart DA (2004) Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: Prominent roles for CYP3A and CYP2D6. *Journal of Pharmacology and Experimental Therapeutics* **310:**1062-1075.

Dhir RN, Dworakowski W, Thangavel C and Shapiro BH (2006) Sexually dimorphic regulation of hepatic isoforms of human cytochrome P450 by growth hormone. *Journal of Pharmacology and Experimental Therapeutics* **316:**87-94.

di Masi A, Marinis ED, Ascenzi P and Marino M (2009) Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects. *Molecular Aspects of Medicine* **30:**297-343.

Dickins M (2004) Induction of cytochromes P450. *Current Topics in Medicinal Chemistry* **4:**1745-1766.

Ding X, Lichti K, Kim I, Gonzalez FJ and Staudinger JL (2006) Regulation of constitutive androstane receptor and its target genes by fasting, cAMP, hepatocyte nuclear factor 4α, and the coactivator peroxisome proliferator-activated receptor γ coactivator-1α. *Journal of Biological Chemistry* **281:**26540-26551.

Ding X and Staudinger JL (2005a) Induction of drug metabolism by forskolin: The role of the pregnane X receptor and the protein kinase A signal transduction pathway. *Journal of Pharmacology and Experimental Therapeutics* **312:**849-856.

Ding X and Staudinger JL (2005b) Repression of PXR-mediated induction of hepatic CYP3A gene expression by protein kinase C. *Biochemical Pharmacology* **69:**867-873.

Dixon JM, Renshaw L, Young O, Murray J, Macaskill EJ, McHugh M, Folkerd E, Cameron DA, A'Hern RP and Dowsett M (2008) Letrozole suppresses plasma estradiol and estrone sulphate more completely than anastrozole in postmenopausal women with breast cancer. *Journal of Clinical Oncology* **26:**1671-1676.

Dong H, Lin W, Wu J and Chen T (2010) Flavonoids activate pregnane X receptor-mediated CYP3A4 gene expression by inhibiting cyclin-dependent kinases in HepG2 liver carcinoma cells. *BMC Biochemistry* **11:**Art. no. 23.

Down MJ, Arkle S and Mills JJ (2007) Regulation and induction of CYP3A11, CYP3A13 and CYP3A25 in C57BL/6J mouse liver. *Archives of Biochemistry and Biophysics* **457:**105-110.

Dowsett M, Cuzick J, Howell A and Jackson I (2001) Pharmacokinetics of anastrozole and tamoxifen alone, and in combination, during adjuvant endocrine therapy for early breast cancer in postmenopausal women: A sub-protocol of the 'Arimidex® and tamoxifen alone or in combination' (ATAC) trial. *British Journal of Cancer* **85:**317-324.

Dowsett M, Jones A, Johnston SRD, Jacobs S, Trunet P and Smith IE (1995) In vivo measurement of aromatase inhibition by letrozole (CGS 20267) in postmenopausal patients with breast cancer. *Clinical Cancer Research* **1:**1511-1515.

Dowsett M, Pfister C, Johnston SRD, Miles DW, Houston SJ, Verbeek JA, Gundacker H, Sioufi A and Smith IE (1999) Impact of tamoxifen on the pharmacokinetics and endocrine effects of the aromatase inhibitor letrozole in postmenopausal women with breast cancer. *Clinical Cancer Research* **5:**2338-2343.

Drocourt L, Ourlin JC, Pascussi JM, Maurel P and Vilarem MJ (2002) Expression of CYP3A4, CYP2B6, and CYP2C9 is regulated by the vitamin D receptor pathway in primary human hepatocytes. *Journal of Biological Chemistry* **277:**25125-25132.

Duckett DR and Cameron MD (2011) Metabolism considerations for kinase inhibitors in cancer treatment. *Expert Opinion on Drug Metabolism and Toxicology* **6:**1175-1193.

Dunne FJ (2009) The 'Natural Health Service': Natural does not mean safe. *Advances in Psychiatric Treatment* **15:**49-56.

Duret C, Daujat-Chavanieu M, Pascussi JM, Pichard-Garcia L, Balaguer P, Fabre JM, Vilarem MJ, Maurel P and Gerbal-Chaloin S (2006) Ketoconazole and miconazole are antagonists of the human glucocorticoid receptor: Consequences on the expression and function of the constitutive androstane receptor and the pregnane X receptor. *Molecular Pharmacology* **70:**329-339.

Dussault I, Lin M, Hollister K, Fan M, Termini J, Sherman MA and Forman BM (2002) A structural model of the constitutive androstane receptor defines novel interactions that mediate ligand-independent activity. *Molecular and Cellular Biology* **22:**5270-5280.

Eastell R, Adams J, Clack G, Howell A, Cuzick J, Mackey J, Beckmann MW and Coleman RE (2010) Long-term effects of anastrozole on bone mineral density: 7-year results from the ATAC trial. *Annals of Oncology* **22:**857-862.

Echchgadda I, Song CS, Oh T, Ahmed M, De La Cruz IJ and Chatterjee B (2007) The xenobiotic-sensing nuclear receptors pregnane X receptor, constitutive androstane receptor, and orphan nuclear receptor hepatocyte nuclear factor 4α in the regulation of human steroid-/bile acid-sulfotransferase. *Molecular Endocrinology* **21:**2099-2111.

Edwards BJ, Raisch DW, Shankaran V, McKoy JM, Gradishar W, Bunta AD, Samaras AT, Boyle SN, Bennett CL, West DP and Guise TA (2011) Cancer therapy associated bone loss: Implications for hip fractures in mid-life women with breast cancer. *Clinical Cancer Research* **17:**560-568.

Eidtmann H, de Boer R, Bundred N, Llombart-Cussac A, Davidson N, Neven P, von Minckwitz G, Miller J, Schenk N and Coleman R (2010) Efficacy of zoledronic acid in postmenopausal women with early breast cancer receiving adjuvant letrozole: 36-month results of the ZO-FAST Study. *Annals of Oncology* **21:**2188-2194.

Ekins S, Chang C, Mani S, Krasowski MD, Reschly EJ, Iyer M, Kholodovych V, Ai N, Welsh WJ, Sinz M, Swaan PW, Patel R and Bachmann K (2007) Human pregnane X receptor antagonists and agonists define molecular requirements for different binding sites. *Molecular Pharmacology* **72:**592-603.

Ellis MJ and Ma C (2007) Letrozole in the neoadjuvant setting: The P024 trial. *Breast Cancer Research and Treatment* **105:**33-43.

Engdal S, Steinsbekk A, Klepp O and Nilsen OG (2008) Herbal use among cancer patients during palliative or curative chemotherapy treatment in Norway. *Supportive Care in Cancer* **16:**763-769.

Engiz O, Berberoglu M, Siklar Z, Bilir P and Ocal G (2009) Treatment of autonomous ovarian follicular cyst with long-term anastrozole therapy. *Indian Journal of Pediatrics* **76:**950-951.

Ewens A, Luo L, Berleth E, Alderfer J, Wollman R, Hafeez BB, Kanter P, Mihich E and Ehrke MJ (2006) Doxorubicin plus interleukin-2 chemoimmunotherapy against breast cancer in mice. *Cancer Research* **66:**5419-5426.

Ewens A, Mihich E and Ehrke MJ (2005) Distant metastasis from subcutaneously grown E0771 medullary breast adenocarcinoma. *Anticancer Research* **25:**3905-3915.

FDA (2012) Guidance for Industry: Drug interaction studies - Study design, data analysis, implications for dosing and labelling recommendations *Draft Guidance*.

Feidt DM, Klein K, Hofmann U, Riedmaier S, Knobeloch D, Thasler WE, Weiss TS, Schwab M and Zanger UM (2010) Profiling induction of cytochrome P450 enzyme activity by statins using a new liquid chromatography-tandem mass spectrometry cocktail assay in human hepatocytes. *Drug Metabolism and Disposition* **38:**1589-1597.

Ferguson SS, Chen Y, LeCluyse EL, Negishi M and Goldstein JA (2005) Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4α. *Molecular Pharmacology* **68:**747-757.

Ferguson SS, Lecluyse EL, Negishi M and Goldstein JA (2002) Regulation of human CYP2C9 by the constitutive androstane receptor: Discovery of a new distal binding site. *Molecular Pharmacology* **62:**737-746.

Finn RD, Henderson CJ, Scott CL and Wolf CR (2009) Unsaturated fatty acid regulation of cytochrome P450 expression via a CAR-dependent pathway. *Biochemical Journal* **417:**43-54.

Fleeman N, Bagust A, Boland A, Dickson R, Dundar Y, Moonan M, Oyee J, Blundell M, Davis H, Armstrong A and Thorp N (2011) Lapatinib and trastuzumab in combination with an aromatase inhibitor for the first-line treatment of metastatic hormone receptor-positive breast cancer which over-expresses human epidermal growth factor 2 (HER2): A systematic review and economic analysis. *Health Technology Assessment* **15:**i-100.

Fontana E, Dansette PM and Poli SM (2005) Cytochrome P450 enzymes mechanism based inhibitors: Common sub-structures and reactivity. *Current Drug Metabolism* **6:**413-454.

Forman BM, Tzameli I, Choi HS, Chen J, Simha D, Seol W, Evans RM and Moore DD (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR-β. *Nature* **395:**612-615.

Frank C, Gonzalez MM, Oinonen C, Dunlop TW and Carlberg C (2003) Characterization of DNA Complexes Formed by the Nuclear Receptor Constitutive Androstane Receptor. *Journal of Biological Chemistry* **278:**43299-43310.

Fraser EA, Henderson CJ and Wolf CR (2012) Transcriptional Regulation of Cytochrome P450 Genes, in: *Encyclopedia of Drug Metabolism and Interactions* (Lyubimov AV ed), pp 1-34, John Wiley & Sons, Inc.

Furet P, Batzl C, Bhatnagar A, Francotte E, Rihs G and Lang M (1993) Aromatase inhibitors: Synthesis, biological activity, and binding mode of azole-type compounds. *Journal of Medicinal Chemistry* **36:**1393-1400.

Gachon F, Olela FF, Schaad O, Descombes P and Schibler U (2006) The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. *Cell Metabolism* **4:**25-36.

Garcia-Segura LM, Veiga S, Sierra A, Melcangi RC and Azcoitia I (2003) Aromatase: A neuroprotective enzyme. *Progress in Neurobiology* **71:**31-41.

Geisler J (2011) Differences between the non-steroidal aromatase inhibitors anastrozole and letrozole- of clinical importance. *British Journal of Cancer* **104:**1059-1066.

Geisler J, Haynes B, Anker G, Dowsett M and Lonning PE (2002) Influence of letrozole and anastrozole on total body aromatization and plasma estrogen levels in postmenopausal breast cancer patients evaluated in a randomized, cross-over study. *Journal of Clinical Oncology* **20:**751-757.

Geisler J, Helle H, Ekse D, Duong NK, Evans DB, Nordbo Y, Aas T and Lonning PE (2008) Letrozole is superior to anastrozole in suppressing breast cancer tissue and plasma estrogen levels. *Clinical Cancer Research* **14:**6330-6335.

Geisler J, King N, Dowsett M, Ottestad L, Lundgren S, Walton P, Kormeset PO and Lonning PE (1996) Influence of anastrozole (Arimidex), a selective, non-steroidal aromatase inhibitor, on in vivo aromatisation and plasma oestrogen levels in postmenopausal women with breast cancer. *British Journal of Cancer* **74:**1286-1291.

Geisler J and Lonning PE (2005) Endocrine effects of aromatase inhibitors and inactivators in vivo: Review of data and method limitations. *Journal of Steroid Biochemistry and Molecular Biology* **95:**75-81.

Ghanbari F, Rowland-Yeo K, Bloomer JC, Clarke SE, Lennard MS, Tucker GT and Rostami-Hodjegan A (2006) A critical evaluation of the experimental design of studies of mechanism based enzyme inhibition, with implications for in vitro-in vivo extrapolation. *Current Drug Metabolism* **7:**315-334.

Gibson GG and Skett GGGP (2001) *Introduction to Drug Metabolism 3rd Ed*. Nelson Thornes.

Gillberg M, Skaanild MT and Friis C (2006) Regulation of gender-dependent CYP2A expression in pigs: Involvement of androgens and CAR. *Basic and Clinical Pharmacology and Toxicology* **98:**480-487.

Goldberg RM, Mabee J, Chan L and Wong S (1996) Drug-drug and drug-disease interactions in the ED: Analysis of a high- risk population. *American Journal of Emergency Medicine* **14:**447-450.

Gonnelli S and Petrioli R (2008) Aromatase inhibitors, efficacy and metabolic risk in the treatment of postmenopausal women with early breast cancer. *Clinical Interventions in Aging* **3:**647-657.

Goodwin B, Hodgson E, D'Costa DJ, Robertson GR and Liddle C (2002) Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor. *Molecular Pharmacology* **62:**359-365.

Goodwin B, Hodgson E and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Molecular Pharmacology* **56:**1329-1339.

Goodwin B, Moore LB, Stoltz CM, McKee DD and Kliewer SA (2001) Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. *Molecular Pharmacology* **60:**427-431.

Goss PE (2007) Letrozole in the extended adjuvant setting: MA.17. *Breast Cancer Research and Treatment* **105:**45-53.

Graham MJ and Lake BG (2008) Induction of drug metabolism: Species differences and toxicological relevance. *Toxicology* **254:**184-191.

Gratus C, Damery S, Wilson S, Warmington S, Routledge P, Grieve R, Steven N, Jones J and Greenfield S (2009a) The use of herbal medicines by people with cancer in the UK: A systematic review of the literature. *QJM* **102:**831-842.

Gratus C, Wilson S, Greenfield SM, Damery SL, Warmington SA, Grieve R, Steven NM and Routledge P (2009b) The use of herbal medicines by people with cancer: A qualitative study. *BMC Complementary and Alternative Medicine* **9**.

Greenblatt DJ and Von Moltke LL (2008) Gender has a small but statistically significant effect on clearance of CYP3A substrate drugs. *Journal of Clinical Pharmacology* **48:**1350-1355.

Gregoriou O, Bakas P, Grigoriadis C, Creatsa M, Hassiakos D and Creatsas G (2012) Changes in hormonal profile and seminal parameters with use of aromatase inhibitors in management of infertile men with low testosterone to estradiol ratios. *Fertility and Sterility* **98:**48-51.

Grimm SW and Dyroff MC (1997) Inhibition of human drug metabolizing cytochromes P450 by anastrozole, a potent and selective inhibitor of aromatase. *Drug Metabolism and Disposition* **25:**598-602.

Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C and Guguen-Guillouzo C (2002) Infection of a human hepatoma cell line by hepatitis B virus. *Proceedings of the National Academy of Sciences of the United States of America* **99:**15655-15660.

Gu JW, Young E, Busby B, Covington J, Tan W and Johnson JW (2009) Oral administration of pyrrolidine dithiocarbamate (PDTC) inhibits VEGF

expression, tumor angiogenesis and growth of breast cancer in female mice. *Cancer Biology and Therapy* **8:**514-521.

Gu JW, Young E, Patterson SG, Makey KL, Wells J, Huang M, Tucker KB and Miele L (2011) Postmenopausal obesity promotes tumor angiogenesis and breast cancer progression in mice. *Cancer Biology and Therapy* **11:**910-917.

Guguen-Guillouzo C and Guillouzo A (2010) General review on in vitro hepatocyte models and their applications. *Methods in molecular biology (Clifton, N.J.)* **640:**1-40.

Guillouzo A, Corlu A, Aninat C, Glaise D, Morel F and Guguen-Guillouzo C (2007) The human hepatoma HepaRG cells: A highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chemico-Biological Interactions* **168:**66-73.

Han S, Choi S, Chun YJ, Yun CH, Lee CH, Shin HJ, Na HS, Chung MW and Kim D (2012) Functional characterization of allelic variants of polymorphic human cytochrome P450 2A6 (CYP2A6*5, *7, *8, *18, *19, and *35). *Biological and Pharmaceutical Bulletin* **35:**394-399.

Hanigan MH, dela Cruz BL, Thompson DM, Farmer KC and Medina PJ (2008) Use of prescription and nonprescription medications and supplements by cancer patients during chemotherapy: Questionnaire validation. *Journal of Oncology Pharmacy Practice* **14:**123-130.

Hariparsad N, Chu X, Yabut J, Labhart P, Hartley DP, Dai X and Evers R (2009) Identification of pregnane-X receptor target genes and coactivator and corepressor binding to promoter elements in human hepatocytes. *Nucleic Acids Research* **37:**1160-1173.

Harmsen S, Meijerman I, Beijnen JH and Schellens JHM (2007) The role of nuclear receptors in pharmacokinetic drug-drug interactions in oncology. *Cancer Treatment Reviews* **33:**369-380.

Harmsen S, Meijerman I, Beijnen JH and Schellens JHM (2009) Nuclear receptor mediated induction of cytochrome P450 3A4 by anticancer drugs: A key role for the pregnane X receptor. *Cancer Chemotherapy and Pharmacology* **64:**35-43.

Harmsen S, Meijerman I, Febus CL, Maas-Bakker RF, Beijnen JH and Schellens JHM (2010) PXR-mediated induction of P-glycoprotein by anticancer drugs in a human colon adenocarcinoma-derived cell line. *Cancer Chemotherapy and Pharmacology* **66:**765-771.

Hasegawa M, Kapelyukh Y, Tahara H, Seibler J, Rode A, Krueger S, Lee DN, Wolf CR and Scheer N (2011) Quantitative prediction of human pregnane X receptor and cytochrome P450 3A4 mediated drug-drug interaction in a novel multiple humanized mouse line. *Molecular Pharmacology* **80:**518-528.

Haynes BP, Dowsett M, Miller WR, Dixon JM and Bhatnagar AS (2003) The pharmacology of letrozole. *Journal of Steroid Biochemistry and Molecular Biology* **87:**35-45.

He SM, Yang AK, Li XT, Du YM and Zhou SF (2010) Effects of herbal products on the metabolism and transport of anticancer agents. *Expert Opinion on Drug Metabolism and Toxicology* **6:**1195-1213.

Henderson CJ, Otto DME, Carrie D, Magnuson MA, McLaren AW, Rosewell I and Wolf CR (2003) Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. *Journal of Biological Chemistry* **278:**13480-13486.

Hernandez JP, Chapman LM, Kretschmer XC and Baldwin WS (2006) Gender-specific induction of cytochrome P450s in nonylphenol-treated FVB/NJ mice. *Toxicology and Applied Pharmacology* **216:**186-196.

Hernandez JP, Huang W, Chapman LM, Chua S, Moore DD and Baldwin WS (2007) The environmental estrogen, nonylphenol, activates the constitutive androstane receptor. *Toxicological Sciences* **98:**416-426.

Hernandez JP, Mota LC and Baldwin WS (2009a) Activation of CAR and PXR by dietary, environmental and occupational chemicals alters drug metabolism,

intermediary metabolism, and cell proliferation. *Current Pharmacogenomics and Personalized Medicine* **7:**81-105.

Hernandez JP, Mota LC, Huang W, Moore DD and Baldwin WS (2009b) Sexually dimorphic regulation and induction of P450s by the constitutive androstane receptor (CAR). *Toxicology* **256:**53-64.

Hisaka A, Ohno Y, Yamamoto T and Suzuki H (2010) Theoretical considerations on quantitative prediction of drug-drug interactions. *Drug Metabolism and Pharmacokinetics* **25:**48-61.

Hoekstra R, Nibourg GAA, Van Der Hoeven TV, Ackermans MT, Hakvoort TBM, Van Gulik TM, Lamers WH, Elferink RPO and Chamuleau RAFM (2011) The HepaRG cell line is suitable for bioartificial liver application. *International Journal of Biochemistry and Cell Biology* **43:**1483-1489.

Hoffart E, Ghebreghiorghis L, Nussler AK, Thasler WE, Weiss TS, Schwab M and Burk O (2011) Effects of atorvastatin metabolites on induction of drug-metabolizing enzymes and membrane transporters through human pregnane X receptor. *British Journal of Pharmacology* **165:**1595-1608.

Hokkanen J, Tolonen A, Mattila S and Turpeinen M (2011) Metabolism of hyperforin, the active constituent of St. John's wort, in human liver microsomes. *European Journal of Pharmaceutical Sciences* **42:**273-284.

Hong Y, Rashid R and Chen S (2011) Binding features of steroidal and nonsteroidal inhibitors. *Steroids* **76:**802-806.

Honkakoski P, Jääskeläinen I, Kortelahti M and Urtti A (2001) A novel drug-regulated gene expression system based on the nuclear receptor constitutive androstane receptor (CAR). *Pharmaceutical Research* **18:**146-150.

Honkakoski P, Sueyoshi T and Negishi M (2003) Drug-activated nuclear receptors CAR and PXR. *Annals of Medicine* **35:**172-182.

Howe K, Sanat F, Thumser AE, Coleman T and Plant N (2011) The statin class of HMG-CoA reductase inhibitors demonstrate differential activation of the

nuclear receptors PXR, CAR and FXR, as well as their downstream target genes. *Xenobiotica* **41:**519-529.

Hrycay EG and Bandiera SM (2009) Expression, function and regulation of mouse cytochrome P450 enzymes: Comparison with human cytochrome P450 enzymes. *Current Drug Metabolism* **10:**1151-1183.

Hu G, Xu C and Staudinger JL (2010) Pregnane X receptor is SUMOylated to repress the inflammatory response. *Journal of Pharmacology and Experimental Therapeutics* **335:**342-350.

Hu TM and Hayton WL (2011) Architecture of the drug-drug interaction network. *Journal of Clinical Pharmacy and Therapeutics* **36:**135-143.

Hu ZY and Zhao YS (2010) Sex-dependent differences in cytochrome P450 3A activity as assessed by midazolam disposition in humans: A meta-analysis. *Drug Metabolism and Disposition* **38:**817-823.

Huang W, Zhang J, Washington M, Liu J, Parant JM, Lozano G and Moore DD (2005) Xenobiotic stress induces hepatomegaly and liver tumors via the nuclear receptor constitutive androstane receptor. *Molecular Endocrinology* **19:**1646-1653.

Hunt CM, Westerkam WR and Stave GM (1992) Effect of age and gender on the activity of human hepatic CYP3A. *Biochemical Pharmacology* **44:**275-283.

Huober J, Fasching PA, Barsoum M, Petruzelka L, Wallwiener D, Thomssen C, Reimer T, Paepke S, Azim HA, Ragosch V, Kubista E, Baumgärtner AK, Beckmann MW, May C, Nimmrich I and Harbeck N (2012) Higher efficacy of letrozole in combination with trastuzumab compared to letrozole monotherapy as first-line treatment in patients with HER2-positive, hormone-receptor-positive metastatic breast cancer - Results of the eLEcTRA trial. *Breast* **21:**27-33.

Ingle JN, Buzdar AU, Schaid DJ, Goetz MP, Batzler A, Robson ME, Northfelt DW, Olson JE, Perez EA, Desta Z, Weintraub RA, Williard CV, Flockhart DA and Weinshilboum RM (2010) Variation in anastrozole metabolism and

pharmacodynamics in women with early breast cancer. *Cancer Research* **70:**3278-3286.

Ingle JN, Suman VJ, Johnson PA, Krook JE, Mailliard JA, Wheeler RH, Loprinzi CL, Perez EA, Jordan VC and Dowsett M (1999) Evaluation of tamoxifen plus letrozole with assessment of pharmacokinetic interaction in postmenopausal women with metastatic breast cancer. *Clinical Cancer Research* **5:**1642-1649.

Inoue K and Negishi M (2008) Nuclear receptor CAR requires early growth response 1 to activate the human cytochrome P450 2B6 gene. *Journal of Biological Chemistry* **283:**10425-10432.

Inoue K and Negishi M (2009) Early growth response 1 loops the CYP2B6 promoter for synergistic activation by the distal and proximal nuclear receptors CAR and HNF4α. *FEBS Letters* **583:**2126-2130.

Inoue SI, Yoshinari K, Sugawara M and Yamazoe Y (2011) Activated sterol regulatory element-binding protein-2 suppresses hepatocyte nuclear factor-4-mediated Cyp3a11 expression in mouse liver. *Molecular Pharmacology* **79:**148-156.

Inoue T, Miki Y, Abe K, Hatori M, Hosaka M, Kariya Y, Kakuo S, Fujimura T, Hachiya A, Honma S, Aiba S and Sasano H (2012) Sex steroid synthesis in human skin in situ: The roles of aromatase and steroidogenic acute regulatory protein in the homeostasis of human skin. *Molecular and Cellular Endocrinology***:***In Press*.

Istrate MA, Nussler AK, Eichelbaum M and Burk O (2010) Regulation of CYP3A4 by pregnane X receptor: The role of nuclear receptors competing for response element binding. *Biochemical and Biophysical Research Communications* **393:**688-693.

Ito K, Utsunomiya H, Niikura H, Yaegashi N and Sasano H (2011) Inhibition of estrogen actions in human gynecological malignancies: New aspects of endocrine therapy for endometrial cancer and ovarian cancer. *Molecular and Cellular Endocrinology* **340:**161-167.

Itoh M, Nakajima M, Higashi E, Yoshida R, Nagata K, Yamazoe Y and Yokoi T (2006) Induction of human CYP2A6 is mediated by the pregnane X receptor with peroxisome proliferator-activated receptor-α coactivator 1α. *Journal of Pharmacology and Experimental Therapeutics* **319:**693-702.

Iveson TJ, Smith IE, Ahern J, Smithers DA, Trunet PF and Dowsett M (1993) Phase I study of the oral nonsteroidal aromatase inhibitor CGS 20267 in healthy postmenopausal women. *Journal of Clinical Endocrinology and Metabolism* **77:**324-331.

Izzo AA (2012) Interactions between herbs and conventional drugs: Overview of the clinical data. *Medical Principles and Practice* **21:**404-428.

Izzo AA and Ernst E (2009) Interactions between herbal medicines and prescribed drugs: An updated systematic review. *Drugs* **69:**1777-1798.

Jackson JP, Ferguson SS, Negishi M and Goldstein JA (2006) Phenytoin induction of the Cyp2c37 gene is mediated by the constitutive androstane receptor. *Drug Metabolism and Disposition* **34:**2003-2010.

Jaffe CA, Kim Turgeon D, Lown K, Demott-Friberg R and Watkins PB (2002) Growth hormone secretion pattern is an independent regulator of growth hormone actions in humans. *American Journal of Physiology - Endocrinology and Metabolism* **283:**E1008-E1015.

Jarukamjorn K, Sakuma T and Nemoto N (2002) Sexual dimorphic expression of mouse hepatic CYP2B: Alterations during development or after hypophysectomy. *Biochemical Pharmacology* **63:**2037-2041.

Jeong S, Woo MM, Flockhart DA and Desta Z (2009) Inhibition of drug metabolizing cytochrome P450s by the aromatase inhibitor drug letrozole and its major oxidative metabolite 4,4'-methanol- bisbenzonitrile in vitro. *Cancer Chemotherapy and Pharmacology* **64:**867-875.

Jin SJ, Jung JA, Cho SH, Kim UJ, Choe S, Ghim JL, Noh YH, Park HJ, Kim JC, Lim HS and Bae KS (2012) The pharmacokinetics of letrozole: Association with key

body mass metrics. *International Journal of Clinical Pharmacology and Therapeutics* **50:**557-565.

Jingxuan W, Qingyuan Z, Shi J, Meiyan F, Xinmei K, Shu Z, Shuling L and Wenhui Z (2009) Immoderate inhibition of estrogen by anastrozole enhances the severity of experimental polyarthritis. *Experimental Gerontology* **44:**398-405.

Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NCO, LeCluyse EL, Lambert MH, Willson TM, Kliewer SA and Moore JT (2000) The pregnane X receptor: A promiscuous xenobiotic receptor that has diverged during evolution. *Molecular Endocrinology* **14:**27-39.

Jossé R, Aninat C, Glaise D, Dumont J, Fessard V, Morel F, Poul JM, Guguen-Guillouzo C and Guillouzo A (2008) Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies. *Drug Metabolism and Disposition* **36:**1111-1118.

Jungermann K and Kietzmann T (1996) Zonation of parenchymal and nonparenchymal metabolism in liver. *Annual Review of Nutrition* **16:**179-203.

Jyrkkärinne J, Windshügel B, Mäkinen J, Ylisirniö M, Peräkylä M, Poso A, Sippl W and Honkakoski P (2005) Amino acids important for ligand specificity of the human constitutive androstane receptor. *Journal of Biological Chemistry* **280:**5960-5971.

Kamdem LK, Liu Y, Stearns V, Kadlubar SA, Ramirez J, Jeter S, Shahverdi K, Ward BA, Ogburn E, Ratain MJ, Flockhart DA and Desta Z (2010) In vitro and in vivo oxidative metabolism and glucuronidation of anastrozole. *British Journal of Clinical Pharmacology* **70:**854-869.

Kamiguchi N, Aoyama E, Okuda T and Moriwaki T (2010) A 96-well plate assay for CYP450 3A induction using cryopreserved human hepatocytes. *Drug Metabolism and Disposition* **38:**1912-1916.

Kamiya A, Inoue Y and Gonzalez FJ (2003) Role of the hepatocyte nuclear factor 4α in control of the pregnane X receptor during fetal liver development. *Hepatology* **37:**1375-1384.

Kanebratt KP and Andersson TB (2008a) Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies. *Drug Metabolism and Disposition* **36:**1444-1452.

Kanebratt KP and Andersson TB (2008b) HepaRG cells as an in vitro model for evaluation of cytochrome P450 induction in humans. *Drug Metabolism and Disposition* **36:**137-145.

Kaneko A, Kato M, Sekiguchi N, Mitsui T, Takeda K and Aso Y (2009) In vitro model for the prediction of clinical CYP3A4 induction using HepaRG cells Prediction of CYP3A4 induction using HepaRG cells. *Xenobiotica* **39:**803-810.

Kanno Y, Aoki S, Mochizuki M, Mori E, Nakahama T and Inouye Y (2005a) Expression of constitutive androstane receptor splice variants in rat liver and lung and their functional properties. *Biological and Pharmaceutical Bulletin* **28:**2058-2062.

Kanno Y, Inoue Y and Inouye Y (2010a) 5-Aminoimidazole-4-carboxamide-1-β-ribofuranoside (AICAR) prevents nuclear translocation of constitutive androstane receptor by AMP-activated protein kinase (AMPK) independent manner. *Journal of Toxicological Sciences* **35:**571-576.

Kanno Y and Inouye Y (2008) Molecular basis of the intracellular localization of the constitutive androstane receptor (CAR). *Journal of Health Science* **54:**261-266.

Kanno Y, Miyazaki Y and Inouye Y (2010b) The nuclear import of the constitutive androstane receptor by importin/Ran-GTP systems. *Biochimica et Biophysica Acta - Molecular Cell Research* **1803:**968-974.

Kanno Y, Suzuki M, Miyazaki Y, Matsuzaki M, Nakahama T, Kurose K, Sawada Ji and Inouye Y (2007) Difference in nucleocytoplasmic shuttling sequences of rat and human constitutive active/androstane receptor. *Biochimica et Biophysica Acta - Molecular Cell Research* **1773:**934-944.

Kanno Y, Suzuki M, Nakahama T and Inouye Y (2005b) Characterization of nuclear localization signals and cytoplasmic retention region in the nuclear

receptor CAR. *Biochimica et Biophysica Acta - Molecular Cell Research* **1745:**215-222.

Kawamoto T, Kakizaki S, Yoshinari K and Negishi M (2000) Estrogen activation of the nuclear orphan receptor CAR (Constitutive Active Receptor) in induction of the mouse Cyp2b10 gene. *Molecular Endocrinology* **14:**1897-1905.

Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Molecular and Cellular Biology* **19:**6318-6322.

Kawana K, Ikuta T, Kobayashi Y, Gotoh O, Takeda K and Kawajiri K (2003) Molecular mechanism of nuclear translocation of an orphan nuclear receptor, SXR. *Molecular Pharmacology* **63:**524-531.

Kelly CM and Buzdar AU (2010) Anastrozole. *Expert Opinion on Drug Safety* **9:**995-1003.

Kenakin T (2004) Principles: Receptor theory in pharmacology. *Trends in Pharmacological Sciences* **25:**186-192.

Kennedy DA and Seely D (2010) Clinically based evidence of drugherb interactions: A systematic review. *Expert Opinion on Drug Safety* **9:**79-124.

Khan AA, Chow ECY, van Loenen-Weemaes AmMA, Porte RJ, Pang KS and Groothuis GMM (2009) Comparison of effects of VDR versus PXR, FXR and GR ligands on the regulation of CYP3A isozymes in rat and human intestine and liver. *European Journal of Pharmaceutical Sciences* **37:**115-125.

Klaassen CD and Aleksunes LM (2010) Xenobiotic, bile acid, and cholesterol transporters: Function and regulation. *Pharmacological Reviews* **62:**1-96.

Kliewer SA, Goodwin B and Willson TM (2002) The nuclear pregnane X receptor: A key regulator of xenobiotic metabolism. *Endocrine Reviews* **23:**687-702.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterström RH, Perlmann T and Lehmann JM (1998) An

orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92:**73-82.

Kobayashi K, Sueyoshi T, Inoue K, Moore R and Negishi M (2003) Cytoplasmic Accumulation of the Nuclear Receptor CAR by a Tetratricopeptide Repeat Protein in HepG2 Cells. *Molecular Pharmacology* **64:**1069-1075.

Kobayashi K, Yamanaka Y, Iwazaki N, Nakajo I, Hosokawa M, Negishi M and Chiba K (2005) Identification of HMG-CoA reductase inhibitors as activators for human, mouse and rat constitutive androstane receptor. *Drug Metabolism and Disposition* **33:**924-929.

Koeberle D, Ruhstaller T, Jost L, Pagani O, Zaman K, Von Moos R, Oehlschlegel C, Crowe S, Pilop C and Thuerlimann B (2011) Combination of trastuzumab and letrozole after resistance to sequential trastuzumab and aromatase inhibitor monotherapies in patients with estrogen receptor-positive, HER-2-positive advanced breast cancer: A proof-of-concept trial (SAKK 23/03). *Endocrine-Related Cancer* **18:**257-264.

Koh KH, Jurkovic S, Yang K, Choi SY, Jung JW, Kim KP, Zhang W and Jeong H (2012) Estradiol induces cytochrome P450 2B6 expression at high concentrations: Implication in estrogen-mediated gene regulation in pregnancy. *Biochemical Pharmacology* **84:**93-103.

Kőhalmy K, Tamási V, Kóbori L, Sárváry E, Pascussi JM, Porrogi P, Rozman D, Prough RA, Meyer UA and Monostory K (2007) Dehydroepiandrosterone induces human CYP2B6 through the constitutive androstane receptor. *Drug Metabolism and Disposition* **35:**1495-1501.

Koike C, Moore R and Negishi M (2005) Localization of the nuclear receptor CAR at the cell membrane of mouse liver. *FEBS Letters* **579:**6733-6736.

Koike C, Moore R and Negishi M (2007) Extracellular signal-regulated kinase is an endogenous signal retaining the nuclear Constitutive Active/androstane Receptor (CAR) in the cytoplasm of mouse primary hepatocytes. *Molecular Pharmacology* **71:**1217-1221.

Kozakai K, Yamada Y, Oshikata M, Kawase T, Suzuki E, Haramaki Y and Taniguchi H (2012) Reliable high-throughput method for inhibition assay of 8 cytochrome P450 isoforms using cocktail of probe substrates and stable isotope-labeled internal standards. *Drug Metabolism and Pharmacokinetics* **27:**520-529.

Krasowski MD, Ni A, Hagey LR and Ekins S (2011) Evolution of promiscuous nuclear hormone receptors: LXR, FXR, VDR, PXR, and CAR. *Molecular and Cellular Endocrinology* **334:**39-48.

Küblbeck J, Jyrkkärinne J, Molnár F, Kuningas T, Patel J, Windshügel B, Nevalainen T, Laitinen T, Sippl W, Poso A and Honkakoski P (2011a) New in vitro tools to study human constitutive androstane receptor (CAR) biology: Discovery and comparison of human CAR inverse agonists. *Molecular Pharmaceutics* **8:**2424-2433.

Küblbeck J, Laitinen T, Jyrkkärinne J, Rousu T, Tolonen A, Abel T, Kortelainen T, Uusitalo J, Korjamo T, Honkakoski P and Molnár F (2011b) Use of comprehensive screening methods to detect selective human CAR activators. *Biochemical Pharmacology* **82:**1994-2007.

Kumar S, Jaiswal B, Negi S and Tyagi RK (2010) Cross-talk between androgen receptor and pregnane and xenobiotic receptor reveals existence of a novel modulatory action of anti-androgenic drugs. *Biochemical Pharmacology* **80:**964-976.

Lamb DC, Waterman MR, Kelly SL and Guengerich FP (2007) Cytochromes P450 and drug discovery. *Current Opinion in Biotechnology* **18:**504-512.

Lamba V, Lamba J, Yasuda K, Strom S, Davila J, Hancock ML, Fackenthal JD, Rogan PK, Ring B, Wrighton SA and Schuetz EG (2003) Hepatic CYP2B6 Expression: Gender and Ethnic Differences and Relationship to CYP2B6 Genotype and CAR (Constitutive Androstane Receptor) Expression. *Journal of Pharmacology and Experimental Therapeutics* **307:**906-922.

Lamba V, Yasuda K, Lamba JK, Assem M, Davila J, Strom S and Schuetz EG (2004) PXR (NR1I2): Splice variants in human tissues, including brain, and

identification of neurosteroids and nicotine as PXR activators. *Toxicology and applied pharmacology* **199:**251-265.

Lambert CB, Spire C, Claude N and Guillouzo A (2009a) Dose- and time-dependent effects of phenobarbital on gene expression profiling in human hepatoma HepaRG cells. *Toxicology and Applied Pharmacology* **234:**345-360.

Lambert CB, Spire C, Renaud MP, Claude N and Guillouzo A (2009b) Reproducible chemical-induced changes in gene expression profiles in human hepatoma HepaRG cells under various experimental conditions. *Toxicology in Vitro* **23:**466-475.

Laurent V, Fraix A, Montier T, Cammas-Marion S, Ribault C, Benvegnu T, Jaffres PA and Loyer P (2010) Highly efficient gene transfer into hepatocyte-like HepaRG cells: New means for drug metabolism and toxicity studies. *Biotechnology Journal* **5:**314-320.

Lazarus P and Sun D (2010) Potential role of UGT pharmacogenetics in cancer treatment and prevention: Focus on tamoxifen and aromatase inhibitors. *Drug Metabolism Reviews* **42:**176-188.

Le Vee M, Jigorel E, Glaise D, Gripon P, Guguen-Guillouzo C and Fardel O (2006) Functional expression of sinusoidal and canalicular hepatic drug transporters in the differentiated human hepatoma HepaRG cell line. *European Journal of Pharmaceutical Sciences* **28:**109-117.

LeCluyse EL (2001) Pregnane X receptor: Molecular basis for species differences in CYP3A induction by xenobiotics. *Chemico-Biological Interactions* **134:**283-289.

Lee MD, Ayanoglu E and Gong L (2006) Drug-induced changes in P450 enzyme expression at the gene expression level: A new dimension to the analysis of drug-drug interactions. *Xenobiotica* **36:**1013-1080.

Lees J and Chan A (2011) Polypharmacy in elderly patients with cancer: Clinical implications and management. *The Lancet Oncology* **12:**1249-1257.

Legendre C, Hori T, Loyer P, Aninat C, Ishida S, Glaise D, Lucas-Clerc C, Boudjema K, Guguen-Guillouzo C, Corlu A and Morel F (2009) Drug-metabolising enzymes are down-regulated by hypoxia in differentiated human hepatoma HepaRG cells: HIF-1α involvement in CYP3A4 repression. *European Journal of Cancer* **45:**2882-2892.

Leite SB, Wilk-Zasadna I, Zaldivar JM, Airola E, Reis-Fernandes MA, Mennecozzi M, Guguen-Guillouzo C, Chesne C, Guillou C, Alves PM and Coecke S (2012) Three-dimensional HepaRG model as an attractive tool for toxicity testing. *Toxicological Sciences* **130:**106-116.

Lemaire G, De Sousa G and Rahmani R (2004) A PXR reporter gene assay in a stable cell culture system: CYP3A4 and CYP2B6 induction by pesticides. *Biochemical Pharmacology* **68:**2347-2358.

Levova K, Moserova M, Nebert DW, Phillips DH, Frei E, Schmeiser HH, Arlt VM and Stiborova M (2012) NAD(P)H:quinone oxidoreductase expression in Cyp1a-knockout and CYP1A-humanized mouse lines and its effect on bioactivation of the carcinogen aristolochic acid I. *Toxicology and Applied Pharmacology*.

Li H, Chen T, Cottrell J and Wang H (2009a) Nuclear translocation of adenoviral-enhanced yellow fluorescent protein-tagged-human constitutive androstane receptor (hCAR): A novel tool for screening hcar activators in human primary hepatocytes. *Drug Metabolism and Disposition* **37:**1098-1106.

Li H, Ferguson SS and Wang H (2010) Synergistically enhanced CYP2B6 inducibility between a polymorphic mutation in CYP2B6 promoter and pregnane X receptor activation. *Molecular Pharmacology* **78:**704-713.

Li Y, Buckley D, Wang S, Klaassen CD and Zhong XB (2009b) Genetic polymorphisms in the TATA box and upstream phenobarbital-responsive enhancer module of the UGT1A1 promoter have combined effects on UDP-glucuronosyltransferase 1A1 transcription mediated by constitutive androstane receptor, pregnane X receptor, or glucocorticoid receptor in human liver. *Drug Metabolism and Disposition* **37:**1978-1986.

Lichti-Kaiser K, Brobst D, Xu C and Staudinger JL (2009a) A systematic analysis of predicted phosphorylation sites within the human pregnane X receptor protein. *Journal of Pharmacology and Experimental Therapeutics* **331:**65-76.

Lichti-Kaiser K and Staudinger JL (2008) The traditional Chinese herbal remedy tian xian activates pregnane X receptor and induces CYP3A gene expression in hepatocytes. *Drug Metabolism and Disposition* **36:**1538-1545.

Lichti-Kaiser K, Xu C and Staudinger JL (2009b) Cyclic AMP-dependent protein kinase signaling modulates Pregnane x receptor activity in a species-specific manner. *Journal of Biological Chemistry* **284:**6639-6649.

Lin JH (2006) CYP induction-mediated drug interactions: In vitro assessment and clinical implications. *Pharmaceutical Research* **23:**1089-1116.

Lin W, Wu J, Dong H, Bouck D, Zeng FY and Chen T (2008) Cyclin-dependent kinase 2 negatively regulates human pregnane X receptor-mediated CYP3A4 gene expression in HepG2 liver carcinoma cells. *Journal of Biological Chemistry* **283:**30650-30657.

Lindley C, Hamilton G, McCune JS, Faucette S, Shord SS, Hawke RL, Wang H, Gilbert D, Jolley S, Yan B and Lecluyse EL (2002) The effect of cyclophosphamide with and without dexamethasone on cytochrome P450 3A4 and 2B6 in human hepatocytes. *Drug Metabolism and Disposition* **30:**814-822.

Lipton A, Demers LM, Harvey HA, Kambic KB, Grossberg H, Brady C, Adlercruetz H, Trunet PF and Santen RJ (1995) Letrozole (CGS 20267): A phase I study of a new potent oral aromatase inhibitor of breast cancer. *Cancer* **75:**2132-2138.

Liu FJ, Song X, Yang D, Deng R and Yan B (2008) The far and distal enhancers in the CYP3A4 gene co-ordinate the proximal promoter in responding similarly to the pregnane X receptor but differentially to hepatocyte nuclear factor-4α. *Biochemical Journal* **409:**243-250.

Liu L, Mugundu GM, Kirby BJ, Samineni D, Desai PB and Unadkat JD (2012) Quantification of human hepatocyte cytochrome P450 enzymes and transporters induced by HIV protease inhibitors using newly validated LC-

MS/MS cocktail assays and RT-PCR. *Biopharmaceutics and Drug Disposition* **33:**207-217.

Liu XD, Xie L, Zhong Y and Li CX (2000) Gender difference in letrozole pharmacokinetics in rats. *Acta Pharmacologica Sinica* **21:**680-684.

Lo R, Burgoon L, MacPherson L, Ahmed S and Matthews J (2010) Estrogen receptor-dependent regulation of CYP2B6 in human breast cancer cells. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms* **1799:**469-479.

Lønning PE (1998) Pharmacological and clinical profile of anastrozole. *Breast Cancer Research and Treatment* **49:**S53-S57.

Lønning PE (2011) The potency and clinical efficacy of aromatase inhibitors across the breast cancer continuum. *Annals of Oncology* **22:**503-514.

Loryan I, Lindqvist M, Johansson I, Hiratsuka M, Van Der Heiden I, Van Schaik RH, Jakobsson J and Ingelman-Sundberg M (2012) Influence of sex on propofol metabolism, a pilot study: Implications for propofol anesthesia. *European Journal of Clinical Pharmacology* **68:**397-406.

Luchetti S, Bossers K, Van de Bilt S, Agrapart V, Morales RR, Frajese GV and Swaab DF (2011) Neurosteroid biosynthetic pathways changes in prefrontal cortex in Alzheimer's disease. *Neurobiology of Aging* **32:**1964-1976.

Luo G, Cunningham M, Kim S, Burn T, Lin J, Sinz M, Hamilton G, Rizzo C, Jolley S, Gilbert D, Downey A, Mudra D, Graham R, Carroll K, Xie J, Madan A, Parkinson A, Christ D, Selling B, Lecluyse E and Gan LS (2002) CYP3A4 induction by drugs: Correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug Metabolism and Disposition* **30:**795-804.

Lutz JD and Isoherranen N (2012) In vitro-to-in vivo predictions of drug-drug interactions involving multiple reversible inhibitors. *Expert Opinion on Drug Metabolism and Toxicology* **8:**449-466.

Lynch C, Pan Y, Li L, Ferguson SS, Xia M, Swaan PW and Wang H (2012) Identification of Novel Activators of Constitutive Androstane Receptor from

FDA-Approved Drugs by Integrated Computational and Biological Approaches. *Pharmaceutical Research* ***In Press*****:**1-13.

Lynch N and Berry D (2007) Differences in perceived risks and benefits of herbal, over-the-counter conventional, and prescribed conventional, medicines, and the implications of this for the safe and effective use of herbal products. *Complementary Therapies in Medicine* **15:**84-91.

Ma X, Cheung C, Krausz KW, Shah YM, Wang T, Idle JR and Gonzalez FJ (2008a) A double transgenic mouse model expressing human pregnane X receptor and cytochrome P450 3A4. *Drug Metabolism and Disposition* **36:**2506-2512.

Ma X, Idle JR and Gonzalez FJ (2008b) The pregnane X receptor: From bench to bedside. *Expert Opinion on Drug Metabolism and Toxicology* **4:**895-908.

Ma X, Shah Y, Cheung C, Guo GL, Feigenbaum L, Krausz KW, Idle JR and Gonzalez FJ (2007) The pregnane X receptor gene-humanized mouse: A model for investigating drug-drug interactions mediated by cytochromes P450 3A. *Drug Metabolism and Disposition* **35:**194-200.

Mackay A, Urruticoechea A, Michael JM, Dexter T, Fenwick K, Ashworth A, Drury S, Larionov A, Young O, White S, Miller WR, Evans DB and Dowsett M (2007) Molecular response to aromatase inhibitor treatment in primary breast cancer. *Breast Cancer Research* **9**.

MacKenzie PI, Hu DG and Gardner-Stephen DA (2010) The regulation of UDP-glucuronosyltransferase genes by tissue-specific and ligand-activated transcription factors. *Drug Metabolism Reviews* **42:**95-105.

Maglich JM, Stoltz CM, Goodwin B, Hawkins-Brown D, Moore JT and Kliewer SA (2002) Nuclear pregnane X receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Molecular Pharmacology* **62:**638-646.

Maglich JM, Watson J, McMillen PJ, Goodwin B, Willson TM and Moore JT (2004) The Nuclear Receptor CAR Is a Regulator of Thyroid Hormone Metabolism during Caloric Restriction. *Journal of Biological Chemistry* **279:**19832-19838.

Mäkinen J, Reinisalo M, Niemi K, Viitala P, Jyrkkärinne J, Chung H, Pelkonen O and Honkakoski P (2003) Dual action of oestrogens on the mouse constitutive androstane receptor. *Biochemical Journal* **376:**465-472.

Mandlekar S, Hong JL and Kong ANT (2006) Modulation of metabolic enzymes by dietary phytochemicals: A review of mechanisms underlying beneficial versus unfavorable effects. *Current Drug Metabolism* **7:**661-675.

Mannel M (2004) Drug interactions with St John's Wort: Mechanisms and clinical implications. *Drug Safety* **27:**773-797.

Manzi SF and Shannon M (2005) Drug interactions - A review. *Clinical Pediatric Emergency Medicine* **6:**93-102.

Marino M, Di Masi A, Trezza V, Pallottini V, Polticelli F and Ascenzi P (2011) Xenosensors CAR and PXR at work: Impact on statin metabolism. *Current Drug Metabolism* **12:**300-311.

Markopoulos C (2010) Managing bone health in women with breast cancer under adjuvant treatment with aromatase inhibitors: Pretreatment bone mineral density is important. *Breast Cancer Research* **12**.

Markopoulos C, Tzoracoleftherakis E, Polychronis A, Venizelos B, Dafni U, Xepapadakis G, Papadiamantis J, Zobolas V, Misitzis J, Kalogerakos K, Sarantopoulou A, Siasos N, Koukouras D, Antonopoulou Z, Lazarou S and Gogas H (2010) Management of anastrozole-induced bone loss in breast cancer patients with oral risedronate: Results from the ARBI prospective clinical trial. *Breast Cancer Research* **12**.

Martínez-Jimínez CP, Jover R, Donato MT, Castell JV and Gómez-Lechón MJ (2007) Transcriptional regulation and expression of CYP3A4 in hepatocytes. *Current Drug Metabolism* **8:**185-194.

Masahiko N and Honkakoski P (2000) Induction of drug metabolism by nuclear receptor CAR: Molecular mechanisms and implications for drug research. *European Journal of Pharmaceutical Sciences* **11:**259-264.

Mason P (2010) Important drug-nutrient interactions. *Proceedings of the Nutrition Society* **69:**551-557.

Matsumura K, Saito T, Takahashi Y, Ozeki T, Kiyotani K, Fujieda M, Yamazaki H, Kunitoh H and Kamataki T (2004) Identification of a Novel Polymorphic Enhancer of the Human CYP3A4 Gene. *Molecular Pharmacology* **65:**326-334.

Mauras N, Bishop K, Merinbaum D, Emeribe U, Agbo F and Lowe E (2009) Pharmacokinetics and pharmacodynamics of anastrozole in pubertal boys with recent-onset gynecomastia. *Journal of Clinical Endocrinology and Metabolism* **94:**2975-2978.

McCloskey EV, Hannon RA, Lakner G, Fraser WD, Clack G, Miyamoto A, Finkelman RD and Eastell R (2007) Effects of third generation aromatase inhibitors on bone health and other safety parameters: Results of an open, randomised, multi-centre study of letrozole, exemestane and anastrozole in healthy postmenopausal women. *European Journal of Cancer* **43:**2523-2531.

McGinnity DF, Zhang G, Kenny JR, Hamilton GA, Otmani S, Stams KR, Haney S, Brassil P, Stresser DM and Riley RJ (2009) Evaluation of multiple in vitro systems for assessment of CYP3A4 induction in drug discovery: Human hepatocytes, pregnane X receptor reporter gene, and Fa2N-4 and HepaRG Cells. *Drug Metabolism and Disposition* **37:**1259-1268.

McKillop D, McCormick AD, Millar A, Miles GS, Phillips PJ and Hutchison M (2005) Cytochrome P450-dependent metabolism of gefitinib. *Xenobiotica* **35:**39-50.

McLay JS, Stewart D, George J, Rore C and Heys SD (2011) Complementary and alternative medicines use by Scottish women with breast cancer. What, why and the potential for drug interactions? *European Journal of Clinical Pharmacology* **68:**811-819.

Meehan RR, Forrester LM, Stevenson K, Hastie ND, Buchmann A, Kunz HW and Wolf CR (1988) Regulation of phenobarbital-inducible cytochrome P-450s in rat and mouse liver following dexamethasone administration and hypophysectomy. *Biochemical Journal* **254:**789-797.

Meijerman I, Beijnen JH and Schellens JHM (2006) Herb-drug interactions in oncology: Focus on mechanisms of induction. *Oncologist* **11:**742-752.

Miao J, Fang S, Bae Y and Kemper JK (2006) Functional inhibitory cross-talk between constitutive androstane receptor and hepatic nuclear factor-4 in hepatic lipid/glucose metabolism is mediated by competition for binding to the DR1 motif and to the common coactivators, GRIP-1 and PGC-1α. *Journal of Biological Chemistry* **281:**14537-14546.

Micheal F, Ganesan M, Saranya S, Sridevi S, Riyasudeen A and Judith MP (2011) An evaluation of relative bioavailability of anastrozole tablets 1 mg in healthy, adult, postmenopausal/surgically sterile Indian female human subjects under fasting conditions. *Journal of Pharmaceutical Sciences and Research* **3:**1334-1337.

Mihich E and Ehrke MJ (2000) Anticancer drugs plus cytokines: Immunodulation based therapies of mouse tumors. *International Journal of Immunopharmacology* **22:**1077-1081.

Miller WR, Bartlett J, Brodie AMH, Brueggemeier RW, Di Salle E, Lonning PE, Llombart A, Maass N, Maudelonde T, Sasano H and Goss PE (2008) Aromatase inhibitors: Are there differences between steroidal and nonsteroidal aromatase inhibitors and do they matter? *Oncologist* **13:**829-837.

Miller WR and Dixon JM (2002) Endocrine and clinical endpoints of exemestane as neoadjuvant therapy. *Cancer Control* **9:**9-15.

Miller WR, Larionov A, Anderson TJ, Evans DB and Dixon JM (2012) Sequential changes in gene expression profiles in breast cancers during treatment with the aromatase inhibitor, letrozole. *Pharmacogenomics Journal* **12:**10-21.

Min G (2010) Estrogen modulates transactivations of SXR-mediated liver X receptor response element and CAR-mediated phenobarbital response element in HepG2 cells. *Experimental and Molecular Medicine* **42:**731-738.

Miranda V, Fede A, Nobuo M, Ayres V, Giglio A, Miranda M and Riechelmann RP (2011) Adverse drug reactions and drug interactions as causes of hospital admission in oncology. *Journal of Pain and Symptom Management* **42:**342-353.

Mnif W, Pascussi JM, Pillon A, Escande A, Bartegi A, Nicolas JC, CavaillÃ¨s V, Duchesne MJ and Balaguer P (2007) Estrogens and antiestrogens activate hPXR. *Toxicology Letters* **170:**19-29.

Mohammadianpanah M, Ashouri Y, Hoseini S, Amadloo N, Talei A, Tahmasebi S, Nasrolahi H, Mosalaei A, Omidvari S, Ansari M and Mosleh-Shirazi MA (2012) The efficacy and safety of neoadjuvant chemotherapy +/- letrozole in postmenopausal women with locally advanced breast cancer: a randomized phase III clinical trial. *Breast Cancer Research and Treatment* **132:**853-861.

Monnier A (2009) Long-term efficacy and safety of letrozole for the adjuvant treatment of early breast cancer in postmenopausal women: A review. *Therapeutics and Clinical Risk Management* **5:**725-738.

Monnier A (2010) FACE: The barefaced facts of AI potency. *Cancer Management and Research* **2:**267-276.

Monostory K and Dvorak Z (2011) Steroid regulation of drug-metabolizing cytochromes P450. *Current Drug Metabolism* **12:**154-172.

Monostory K and Pascussi JM (2008) Regulation of Drug-metabolizing Human Cytochrome P450s. *Acta Chimica Slovenica* **55:**20-37.

Montville CP, Khabbaz M, Aubuchon M, Williams DB and Thomas MA (2010) Luteal support with intravaginal progesterone increases clinical pregnancy rates in women with polycystic ovary syndrome using letrozole for ovulation induction. *Fertility and Sterility* **94:**678-683.

Moore DD (2005) CAR: Three new models for a problem child. *Cell Metabolism* **1:**6-8.

Moore JT and Kliewer SA (2000) Use of the nuclear receptor PXR to predict drug interactions. *Toxicology* **153:**1-10.

Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL and Kliewer SA (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *Journal of Biological Chemistry* **275:**15122-15127.

Moreau A, Maurel P, Vilarem MJ and Pascussi JM (2007) Constitutive androstane receptor-vitamin D receptor crosstalk: Consequence on CYP24 gene expression. *Biochemical and Biophysical Research Communications* **360:**76-82.

Moreland JL, Gramada A, Buzko OV, Zhang Q and Bourne PE (2005) The Molecular Biology Toolkit (MBT): A modular platform fro developing molecular visualization applications. *BMC Bioinformatics* **6:**21.

Morris ME, Lee HJ and Predko LM (2003) Gender differences in the membrane transport of endogenous and exogenous compounds. *Pharmacological Reviews* **55:**229-240.

Mota LC, Barfield C, Hernandez JP and Baldwin WS (2011) Nonylphenol-mediated CYP induction is PXR-dependent: The use of humanized mice and human hepatocytes suggests that hPXR is less sensitive than mouse PXR to nonylphenol treatment. *Toxicology and Applied Pharmacology* **252:**259-267.

Mouridsen HT (2007) Letrozole in advanced breast cancer: The PO25 trial. *Breast Cancer Research and Treatment* **105:**19-29.

Mouridsen HT and Bhatnagar AS (2005) Letrozole in the treatment of breast cancer. *Expert Opinion on Pharmacotherapy* **6:**1389-1399.

Mu Y, Zhang J, Zhang S, Zhou HH, Toma D, Ren S, Huang L, Yaramus M, Baum A, Venkataramanan R and Xie W (2006) Traditional Chinese medicines Wu Wei Zi (Schisandra chinensis Baill) and Gan Cao (Glycyrrhiza uralensis Fisch) activate pregnane X receptor and increase warfarin clearance in rats. *Journal of Pharmacology and Experimental Therapeutics* **316:**1369-1377.

Muntané J (2009) Regulation of drug metabolism and transporters. *Current Drug Metabolism* **10:**932-945.

Murai K, Yamazaki H, Nakagawa K, Kawai R and Kamataki T (2009) Deactivation of anti-cancer drug letrozole to a carbinol metabolite by polymorphic cytochrome P450 2A6 in human liver microsomes. *Xenobiotica* **39:**795-802.

Mutoh S, Osabe M, Inoue K, Moore R, Pedersen L, Perera L, Rebolloso Y, Sueyoshi T and Negishi M (2009) Dephosphorylation of threonine 38 Is required for nuclear translocation and activation of human xenobiotic receptor CAR (NR1I3). *Journal of Biological Chemistry* **284:**34785-34792.

Nakata K, Tanaka Y, Nakano T, Adachi T, Tanaka H, Kaminuma T and Ishikawa T (2006) Nuclear receptor-mediated transcriptional regulation in Phase I, II, and III xenobiotic metabolizing systems. *Drug metabolism and pharmacokinetics* **21:**437-457.

Nelson DR, Zeldin DC, Hoffman SMG, Maltais LJ, Wain HM and Nebert DW (2004) Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics* **14:**1-18.

Ngô C, Nicco C, Leconte M, Chéreau C, Weill B, Batteux F and Chapron C (2010) Antiproliferative effects of anastrozole, methotrexate, and 5-fluorouracil on endometriosis in vitro and in vivo. *Fertility and Sterility* **94:**1632-1638.e1631.

Nibourg GA, Boer JD, van der Hoeven TV, Ackermans MT, van Gulik TM, Chamuleau RA and Hoekstra R (2012a) Perfusion flow rate substantially contributes to the performance of the HepaRG-AMC-bioartificial liver. *Biotechnology and Bioengineering* **109:**3182-3188.

Nibourg GAA, Chamuleau RAFM, van der Hoeven TV, Maas MAW, Ruiter AFC, Lamers WH, Elferink RPJO, van Gulik TM and Hoekstra R (2012b) Liver progenitor cell line HepaRG differentiated in a bioartificial liver effectively supplies liver support to rats with acute liver failure. *PLoS ONE* **7**.

Nibourg GAA, Chamuleau RAFM, Van Gulik TM and Hoekstra R (2012c) Proliferative human cell sources applied as biocomponent in bioartificial livers: A review. *Expert Opinion on Biological Therapy* **12:**905-921.

Nicolas JM, Espie P and Molimard M (2009) Gender and interindividual variability in pharmacokinetics. *Drug Metabolism Reviews* **41:**408-421.

Noh YH, Ko YJ, Cho SH, Ghim JL, Choe S, Jung JA, Kim UJ, Jin SJ, Park HJ, Song GS, Lim HS and Bae KS (2012) Pharmacokinetic Comparison of 2 Formulations of Anastrozole (1 mg) in Healthy Korean Male Volunteers: A Randomized, Single-Dose, 2-Period, 2-Sequence, Crossover Study. *Clinical Therapeutics* **34:**305-313.

Nothnick WB (2011) The emerging use of aromatase inhibitors for endometriosis treatment. *Reproductive Biology and Endocrinology* **9**.

Nukaya M, Moran S and Bradfield CA (2009) The role of the dioxin-responsive element cluster between the Cyp1a1 and Cyp1a2 loci in aryl hydrocarbon receptor biology. *Proceedings of the National Academy of Sciences of the United States of America* **106:**4923-4928.

Nuzzo F, Gallo C, Lastoria S, Di Maio M, Piccirillo MC, Gravina A, Landi G, Rossi E, Pacilio C, Labonia V, Di Rella F, Bartiromo A, Buonfanti G, De Feo G, Esposito G, D'Aniello R, Maiolino P, Signoriello S, De Maio E, Tinessa V, Colantuoni G, De Laurentiis M, D'Aiuto M, Di Bonito M, Botti G, Giordano P, Daniele G, Morabito A, Normanno N, De Matteis A and Perrone F (2012) Bone effect of adjuvant tamoxifen, letrozole or letrozole plus zoledronic acid in early-stage breast cancer: The randomized phase 3 hoboe study. *Annals of Oncology* **23:**2027-2033.

Obreli-Neto PR, Nobili A, de Oliveira Baldoni A, Guidoni CM, de Lyra Júnior DP, Pilger D, Duzanski J, Tettamanti M, Cruciol-Souza JM, Gaeti WP and Cuman RKN (2012) Adverse drug reactions caused by drug-drug interactions in elderly outpatients: a prospective cohort study. *European Journal of Clinical Pharmacology* ***In Press*****:**1-10.

Olaku O and White JD (2011) Herbal therapy use by cancer patients: A literature review on case reports. *European Journal of Cancer* **47:**508-514.

Omiecinski CJ, Coslo DM, Chen T, Laurenzana EM and Peffer RC (2011a) Multi-species analyses of direct activators of the constitutive androstane receptor. *Toxicological Sciences* **123:**550-562.

Omiecinski CJ, Vanden Heuvel JP, Perdew GH and Peters JM (2011b) Xenobiotic metabolism, disposition, and regulation by receptors: From biochemical phenomenon to predictors of major toxicities. *Toxicological Sciences* **120:**S49-S75.

Orans J, Teotico DG and Redinbo MR (2005) The nuclear xenobiotic receptor pregnane X receptor: Recent insights and new challenges. *Molecular Endocrinology* **19:**2891-2900.

Osabe M and Negishi M (2011) Active ERK1/2 protein interacts with the phosphorylated nuclear constitutive active/androstane receptor (CAR; NR1I3), repressing dephosphorylation and sequestering car in the cytoplasm. *Journal of Biological Chemistry* **286:**35763-35769.

Östberg T, Bertilsson G, Jendeberg L, Berkenstam A and Uppenberg J (2002) Identification of residues in the PXR ligand binding domain critical for species specific and constitutive activation. *European Journal of Biochemistry* **269:**4896-4904.

Pandya N and Morris GJ (2006) Toxicity of Aromatase Inhibitors. *Seminars in Oncology* **33:**688-695.

Papanikolaou EG, Polyzos NP, Humaidan P, Pados G, Bosch E, Tournaye H and Tarlatzis B (2011) Aromatase inhibitors in stimulated IVF cycles. *Reproductive Biology and Endocrinology* **9**.

Parkinson A, Mudra DR, Johnson C, Dwyer A and Carroll KM (2004) The effects of gender, age, ethnicity, and liver cirrhosis on cytochrome P450 enzyme activity in human liver microsomes and inducibility in cultured human hepatocytes. *Toxicology and Applied Pharmacology* **199:**193-209.

Pascussi J-M, Gerbal-Chaloin S, Fabre J-M, Maurel P and Vilarem M-J (2000) Dexamethasone Enhances Constitutive Androstane Receptor Expression in Human Hepatocytes: Consequences on Cytochrome P450 Gene Regulation. *Molecular Pharmacology* **58:**1441-1450.

Pascussi JM, Gerbal-Chaloin S, Drocourt L, Maurel P and Vilarem MJ (2003) The expression of CYP2B6, CYP2C9 and CYP3A4 genes: A tangle of networks of nuclear and steroid receptors. *Biochimica et Biophysica Acta - General Subjects* **1619:**243-253.

Pascussi JM, Gerbal-Chaloin S, Duret C, Daujat-Chavanieu M, Vilarem MJ and Maurel P (2008) The tangle of nuclear receptors that controls xenobiotic metabolism and transport: Crosstalk and consequences, in: *Annual Review of Pharmacology and Toxicology*, pp 1-32.

Pavek P, Pospechova K, Svecova L, Syrova Z, Stejskalova L, Blazkova J, Dvorak Z and Blahos J (2010) Intestinal cell-specific vitamin D receptor (VDR)-mediated transcriptional regulation of CYP3A4 gene. *Biochemical Pharmacology* **79:**277-287.

Perlmann T and Jansson L (1995) A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. *Genes and Development* **9:**769-782.

Petrick JS and Klaassen CD (2007) Importance of hepatic induction of constitutive androstane receptor and other transcription factors that regulate xenobiotic metabolism and transport. *Drug Metabolism and Disposition* **35:**1806-1815.

Pfister CU, Martoni A, Zamagni C, Lelli G, De Braud F, Souppart C, Duval M and Hornberger U (2001) Effect of age and single versus multiple dose pharmacokinetics of letrozole (Femara®) in breast cancer patients. *Biopharmaceutics and Drug Disposition* **22:**191-197.

Pinto N and Dolan ME (2011) Clinically relevant genetic variations in drug metabolizing enzymes. *Current Drug Metabolism* **12:**487-497.

Pirmohamed M, James S, Meakin S, Green C, Scott AK, Walley TJ, Farrar K, Park BK and Breckenridge AM (2004) Adverse drug reactions as cause of admission to hospital: Prospective analysis of 18 820 patients. *British Medical Journal* **329:**15-19.

Piton A, Rauch C, Langouet S, Guillouzo A and Morel F (2010) Involvement of pregnane X receptor in the regulation of CYP2B6 gene expression by oltipraz in human hepatocytes. *Toxicology in Vitro* **24:**452-459.

Plant N (2007) The human cytochrome P450 sub-family: Transcriptional regulation, inter-individual variation and interaction networks. *Biochimica et Biophysica Acta - General Subjects* **1770:**478-488.

Plant N and Aouabdi S (2009) Nuclear receptors: The controlling force in drug metabolism of the liver? *Xenobiotica* **39:**597-605.

Plant NJ, Ogg M, Crowder M and Gibson GG (2000) Control and statistical analysis of in vitro reporter gene assays. *Analytical Biochemistry* **278:**170-174.

Plourde PV, Dyroff M, Dowsett M, Demers L, Yates R and Webster A (1995) ARIMIDEX®: A new oral, once-a-day aromatase inhibitor. *Journal of Steroid Biochemistry and Molecular Biology* **53:**175-179.

Plourde PV, Dyroff M and Dukes M (1994) Arimidex®: A potent and selective fourth-generation aromatase inhibitor. *Breast Cancer Research and Treatment* **30:**103-111.

Polyzos NP, Fatemi HM, Zavos A, Grimbizis G, Kyrou D, Velasco JG, Devroey P, Tarlatzis B and Papanikolaou EG (2011) Aromatase inhibitors in post-menopausal endometriosis. *Reproductive Biology and Endocrinology* **9**.

Pondugula SR, Brimer-Cline C, Wu J, Schuetz EG, Tyagi RK and Chen T (2009) A phosphomimetic mutation at threonine-57 abolishes transactivation activity and alters nuclear localization pattern of human pregnane X receptor. *Drug Metabolism and Disposition* **37:**719-730.

Poso A and Honkakoski P (2006) Ligand recognition by drug-activated nuclear receptors PXR and CAR: Structural, site-directed mutagenesis and molecular modeling studies. *Mini-Reviews in Medicinal Chemistry* **6:**937-943.

Precht JC, Ganchev B, Heinkele G, Brauch H, Schwab M and Mürdter TE (2012) Simultaneous quantitative analysis of letrozole, its carbinol metabolite, and

carbinol glucuronide in human plasma by LC-MS/MS. *Analytical and Bioanalytical Chemistry* **403:**301-308.

Prithviraj GK, Koroukian S, Margevicius S, Berger NA, Bagai R and Owusu C (2012) Patient characteristics associated with polypharmacy and inappropriate prescribing of medications among older adults with cancer. *Journal of Geriatric Oncology* **3:**228-237.

Pritts EA (2011) Letrozole for ovulation induction and controlled ovarian hyperstimulation. *Current Opinion in Obstetrics and Gynecology* **22:**289-294.

Prot JM, Videau O, Brochot C, Legallais C, Bénech H and Leclerc E (2011) A cocktail of metabolic probes demonstrates the relevance of primary human hepatocyte cultures in a microfluidic biochip for pharmaceutical drug screening. *International Journal of Pharmaceutics* **408:**67-75.

Purnapatre K, Khattar SK and Saini KS (2008) Cytochrome P450s in the development of target-based anticancer drugs. *Cancer Letters* **259:**1-15.

Pustylnyak VO, Gulyaeva LF and Lyakhovich VV (2007) Induction of cytochrome P4502B: Role of regulatory elements and nuclear receptors. *Biochemistry (Moscow)* **72:**608-617.

Puts MTE, Costa-Lima B, Monette J, Girre V, Wolfson C, Batist G and Bergman H (2009) Medication problems in older, newly diagnosed cancer patients in canada: How common are they?: A prospective pilot study. *Drugs and Aging* **26:**519-536.

Quattrochi LC and Guzelian PS (2001) CYP3A regulation: From pharmacology to nuclear receptors. *Drug Metabolism and Disposition* **29:**615-622.

Rana R, Coulter S, Kinyamu H and Goldstein JA (2012) RBCK1, an E3 Ubiquitin Ligase, Interacts with and Ubiquinates the Human Pregnane X Receptor (hPXR). *Drug Metabolism and Disposition* ***In Press***.

Raucy J, Warfe L, Yueh MF and Allen SW (2002) A cell-based reporter gene assay for determining induction of CYP3A4 in a high-volume system. *Journal of Pharmacology and Experimental Therapeutics* **303:**412-423.

Raunio H, Rautio A, Gullstén H and Pelkonen O (2001) Polymorphisms of CYP2A6 and its practical consequences. *British Journal of Clinical Pharmacology* **52:**357-363.

Rautio A (2003) Polymorphic CYP2A6 and its clinical and toxicological significance. *Pharmacogenomics Journal* **3:**5-7.

Regan MM, Giobbie-Hurder A, Price KN, Thürlimann B, Gelber RD, International Breast Cancer Study G and Group BIGC (2011a) Interpreting Breast International Group (BIG) 1-98: a randomized, double-blind, phase III trial comparing letrozole and tamoxifen as adjuvant endocrine therapy for postmenopausal women with hormone receptor-positive, early breast cancer. *Breast cancer research : BCR* **13:**209.

Regan MM, Neven P, Giobbie-Hurder A, Goldhirsch A, Ejlertsen B, Mauriac L, Forbes JF, Smith I, Láng I, Wardley A, Rabaglio M, Price KN, Gelber RD, Coates AS and Thürlimann B (2011b) Assessment of letrozole and tamoxifen alone and in sequence for postmenopausal women with steroid hormone receptor-positive breast cancer: The BIG 1-98 randomised clinical trial at 8·1 years median follow-up. *The Lancet Oncology* **12:**1101-1108.

Reis AMM and Cassiani SHDB (2011) Adverse drug events in an intensive care unit of a university hospital. *European Journal of Clinical Pharmacology* **67:**625-632.

Rencurel F, Foretz M, Kaufmann MR, Stroka D, Looser R, Leclerc I, Xavier GDS, Rutter GA, Viollet B and Meyer UA (2006) Stimulation of AMP-activated protein kinase is essential for the induction of drug metabolizing enzymes by phenobarbital in human and mouse liver. *Molecular Pharmacology* **70:**1925-1934.

Rencurel F, Stenhouse A, Hawley SA, Friedberg T, Hardie DG, Sutherland C and Wolf CR (2005) AMP-activated protein kinase mediates phenobarbital induction of CYP2B gene expression in hepatocytes and a newly derived human hepatoma cell line. *Journal of Biological Chemistry* **280:**4367-4373.

Repo S, Jyrkkärinne J, Pulkkinen JT, Laatikainen R, Honkakoski P and Johnson MS (2008) Ligand specificity of constitutive androstane receptor as probed by induced-fit docking and mutagenesis. *Journal of Medicinal Chemistry* **51:**7119-7131.

Rezen T, Rozman D, Pascussi JM and Monostory K (2010) Interplay between cholesterol and drug metabolism. *Biochimica et Biophysica Acta - Proteins and Proteomics* **1814:**146-160.

Rhodes SP, Otten JN, Hingorani GP, Hartley DP and Franklin RB (2011) Simultaneous assessment of cytochrome P450 activity in cultured human hepatocytes for compound-mediated induction of CYP3A4, CYP2B6, and CYP1A2. *Journal of Pharmacological and Toxicological Methods* **63:**223-226.

Ricketts ML, Boekschoten MV, Kreeft AJ, Hooiveld GJEJ, Moen CJA, Müller M, Frants RR, Kasanmoentalib S, Post SM, Princen HMG, Porter JG, Katan MB, Hofker MH and Moore DD (2007) The cholesterol-raising factor from coffee beans, cafestol, as an agonist ligand for the farnesoid and pregnane X receptors. *Molecular Endocrinology* **21:**1603-1616.

Riechelmann RP (2007) Drug combinations with the potential to interact among cancer patients [1]. *Supportive Care in Cancer* **15:**1113-1114.

Riechelmann RP and Del Giglio A (2009) Drug interactions in oncology: How common are they? *Annals of Oncology* **20:**1907-1912.

Riechelmann RP and Krzyzanowska MK (2006) Epidemiology of drug interactions in cancer patients [1]. *Nature Reviews Cancer* **6:**1.

Riechelmann RP and Krzyzanowska MK (2007) Issues of potential drug interactions and prescription duplication among patients with cancer. *American Journal of Hematology/ Oncology* **6:**529-531.

Riechelmann RP, Moreira F, Smaletz Ò and Saad ED (2005) Potential for drug interactions in hospitalized cancer patients. *Cancer Chemotherapy and Pharmacology* **56:**286-290.

Riechelmann RP and Saad ED (2006) A systematic review on drug interactions in oncology. *Cancer Investigation* **24:**704-712.

Riechelmann RP, Zimmermann C, Chin SN, Wang L, O'Carroll A, Zarinehbaf S and Krzyzanowska MK (2008) Potential Drug Interactions in Cancer Patients Receiving Supportive Care Exclusively. *Journal of Pain and Symptom Management* **35:**535-543.

Robertson GR, Field J, Goodwin B, Bierach S, Tran M, Lehnert A and Liddle C (2003) Transgenic mouse models of human CYP3A4 gene regulation. *Molecular Pharmacology* **64:**42-50.

Rockwell S, Liu Y and Higgins SA (2005) Alteration of the effects of cancer therapy agents on breast cancer cells by the herbal medicine black cohosh. *Breast Cancer Research and Treatment* **90:**233-239.

Rodríguez-Antona C, Bort R, Jover R, Tindberg N, Ingelman-Sundberg M, Gómez-Lechón MJ and Castell JV (2003) Transcriptional regulation of human CYP3A4 basal expression by CCAAT enhancer-binding protein α and hepatocyte nuclear factor-3γ. *Molecular Pharmacology* **63:**1180-1189.

Ross J, Plummer SM, Rode A, Scheer N, Bower CC, Vogel O, Henderson CJ, Wolf CR and Elcombe CR (2010) Human constitutive androstane receptor (CAR) and pregnane X receptor (PXR) support the hypertrophic but not the hyperplastic response to the murine nongenotoxic hepatocarcinogens phenobarbital and chlordane in vivo. *Toxicological Sciences* **116:**452-466.

Roth A, Looser R, Kaufmann M, BlÃ¤ttler SM, Rencurel F, Huang W, Moore DD and Meyer UA (2008) Regulatory cross-talk between drug metabolism and lipid homeostasis: Constitutive androstane receptor and pregnane X receptor increase Insig-1 expression. *Molecular Pharmacology* **73:**1282-1289.

Rulcova A, Prokopova I, Krausova L, Bitman M, Vrzal R, Dvorak Z, Blahos J and Pavek P (2010) Stereoselective interactions of warfarin enantiomers with the pregnane X nuclear receptor in gene regulation of major drug-metabolizing

cytochrome P450 enzymes. *Journal of Thrombosis and Haemostasis* **8:**2708-2717.

Sahi J, Shord SS, Lindley C, Ferguson S and LeCluyse EL (2009) Regulation of cytochrome P450 2C9 expression in primary cultures of human hepatocytes. *Journal of Biochemical and Molecular Toxicology* **23:**43-58.

Saini SPS, Sonoda J, Xu L, Toma D, Uppal H, Mu Y, Ren S, Moore DD, Evans RM and Xie W (2004) A Novel Constitutive Androstane Receptor-Mediated and CYP3A-Independent Pathway of Bile Acid Detoxification. *Molecular Pharmacology* **65:**292-300.

Sane RS, Buckley DJ, Buckley AR, Nallani SC and Desai PB (2008a) Role of human pregnane X receptor in tamoxifen- and 4-hydroxytamoxifen- mediated CYP3A4 induction in primary human hepatocytes and LS174T cells. *Drug Metabolism and Disposition* **36:**946-954.

Sane RS, Buckley DJ, Buckley AR, Nallani SC and Desai PB (2008b) Role of human pregnane X receptor in tamoxifenand 4-hydroxytamoxifen- mediated CYP3A4 induction in primary human hepatocytes and LS174T cells. *Drug Metabolism and Disposition* **36:**946-954.

Sanford M and Plosker GL (2008) Anastrozole: A review of its use in postmenopausal women with early-stage breast cancer. *Drugs* **68:**1319-1340.

Saradhi M, Sengupta A, Mukhopadhyay G and Tyagi RK (2005) Pregnane and Xenobiotic Receptor (PXR/SXR) resides predominantly in the nuclear compartment of the interphase cell and associates with the condensed chromosomes during mitosis. *Biochimica et Biophysica Acta - Molecular Cell Research* **1746:**85-94.

Satsu H, Hiura Y, Mochizuki K, Hamada M and Shimizu M (2008) Activation of pregnane X receptor and induction of MDR1 by dietary phytochemicals. *Journal of Agricultural and Food Chemistry* **56:**5366-5373.

Saylam B, Efesoy O and Ayan S (2011) The effect of aromatase inhibitor letrozole on body mass index, serum hormones, and sperm parameters in infertile men. *Fertility and Sterility* **95:**809-811.

Schech AJ, Nemieboka BE and Brodie AH (2012) Zoledronic acid inhibits aromatase activity and phosphorylation: Potential mechanism for additive zoledronic acid and letrozole drug interaction. *Journal of Steroid Biochemistry and Molecular Biology* **132:**195-202.

Scheer N, Kapelyukh Y, McEwan J, Beuger V, Stanley LA, Rode A and Wolf CR (2012) Modeling human cytochrome P450 2D6 metabolism and drug-drug interaction by a novel panel of knockout and humanized mouse lines. *Molecular Pharmacology* **81:**63-72.

Scheer N, Ross J, Kapelyukh Y, Rode A and Wolf CR (2010) In vivo responses of the human and murine pregnane X receptor to dexamethasone in mice. *Drug Metabolism and Disposition* **38:**1046-1053.

Scheer N, Ross J, Rode A, Zevnik B, Niehaves S, Faust N and Wolf CR (2008) A novel panel of mouse models to evaluate the role of human pregnane X receptor and constitutive androstane receptor in drug response. *Journal of Clinical Investigation* **118:**3228-3239.

Schuetz EG, Schmid W, Schutz G, Brimer C, Yasuda K, Kamataki T, Bornheim L, Myles K and Cole TJ (2000) The glucocorticoid receptor is essential for induction of cytochrome P- 4502B by steroids but not for drug or steroid induction of CYP3A or P-450 reductase in mouse liver. *Drug Metabolism and Disposition* **28:**268-278.

Schwartz JB (2007) The current state of knowledge on age, sex, and their interactions on clinical pharmacology. *Clinical Pharmacology and Therapeutics* **82:**87-96.

Scripture CD, Sparreboom A and Figg WD (2005) Modulation of cytochrome P450 activity: Implications for cancer therapy. *Lancet Oncology* **6:**780-789.

Seal SL, Kamilya G, Mukherji J, De A, Ghosh D and Majhi AK (2011) Aromatase inhibitors in recurrent ovarian endometriomas: Report of five cases with literature review. *Fertility and Sterility* **95:**291.e215-291.e218.

Sestak I, Distler W, Forbes JF, Dowsett M, Howell A and Cuzick J (2010) Effect of body mass index on recurrences in tamoxifen and anastrozole treated women: An exploratory analysis from the ATAC trial. *Journal of Clinical Oncology* **28:**3411-3415.

Sherrill B, Amonkar MM, Sherif B, Maltzman J, O'Rourke L and Johnston S (2010) Quality of life in hormone receptor-positive HER-2 + metastatic breast cancer patients during treatment with letrozole alone or in combination with lapatinib. *Oncologist* **15:**944-953.

Shindo S, Numazawa S and Yoshida T (2007) A physiological role of AMP-activated protein kinase in phenobarbital-mediated constitutive androstane receptor activation and CYP2B induction. *Biochemical Journal* **401:**735-741.

Shiratani H, Katoh M, Nakajima M and Yokoi T (2008) Species differences in UDP-glucuronosyltransferase activities in mice and rats. *Drug Metabolism and Disposition* **36:**1745-1752.

Shukla SJ, Sakamuru S, Huang R, Moeller TA, Shinn P, Van Leer D, Auld DS, Austin CP and Xia M (2011) Identification of clinically used drugs that activate pregnane X receptors. *Drug Metabolism and Disposition* **39:**151-159.

Singh D, Kashyap A, Pandey RV and Saini KS (2011) Novel advances in cytochrome P450 research. *Drug Discovery Today* **16:**793-799.

Sinz M, Kim S, Zhu Z, Chen T, Anthony M, Dickinson K and Rodrigues AD (2006) Evaluation of 170 xenobiotics as transactivators of human pregnane X receptor (hPXR) and correlation to known CYP3A4 drug interactions. *Current Drug Metabolism* **7:**375-388.

Sinz M, Wallace G and Sahi J (2008) Current industrial practices in assessing CYP450 enzyme induction: Preclinical and clinical. *AAPS Journal* **10:**391-400.

Sioufi A, Gauducheau N, Pineau V, Marfil F, Jaouen A, Cardot JM, Godbillon J, Czendlik C, Howald H, Pfister CH and Vreeland F (1997a) Absolute bioavailability of letrozole in healthy postmenopausal women. *Biopharmaceutics and Drug Disposition* **18:**779-789.

Sioufi A, Sandrenan N, Godbillon J, Trunet P, Czendlik C, Howald H, Pfister C and Ezzet F (1997b) Comparative bioavailability of letrozole under fed and fasting conditions in 12 healthy subjects after a 2.5 mg single oral administration. *Biopharmaceutics and Drug Disposition* **18:**489-497.

Sirotnak FM, Otter GM and Schmid FA (1993) Markedly improved efficacy of edatrexate compared to methotrexate in a high-dose regimen with leucovorin rescue against metastatic murine solid tumors. *Cancer Research* **53:**587-591.

Song N-N, Zhang S-Y and Liu C-X (2004) Overview of factors affecting oral drug absorption. *Asian Journal of Drug Metabolism and Pharmacokinetics* **4:**167-176.

Sönmezer M, Türküolu I, Cokun U and Oktay K (2011) Random-start controlled ovarian hyperstimulation for emergency fertility preservation in letrozole cycles. *Fertility and Sterility* **95:**2125.e2129-2225.e2111.

Squires EJ, Sueyoshi T and Negishi M (2004) Cytoplasmic localization of pregnane X receptor and ligand-dependent nuclear translocation in mouse liver. *Journal of Biological Chemistry* **279:**49307-49314.

Stagg J, Divisekera U, McLaughlin N, Sharkey J, Pommey S, Denoyer D, Dwyer KM and Smyth MJ (2010) Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. *Proceedings of the National Academy of Sciences of the United States of America* **107:**1547-1552.

Stanley L, Horsburgh B, Ross J, Scheer N and Roland Wolf C (2006) PXR and CAR: Nuclear receptors which play a pivotal role in drug disposition and chemical toxicity. *Drug Metabolism Reviews* **38:**515-597.

Staudinger JL, Ding X and Lichti K (2006) Pregnane X receptor and natural products: Beyond drug-drug interactions. *Expert Opinion on Drug Metabolism and Toxicology* **2:**847-857.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH and Kliewer SA (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proceedings of the National Academy of Sciences of the United States of America* **98:**3369-3374.

Staudinger JL, Xu C, Biswas A and Mani S (2011) Post-translational modification of pregnane x receptor. *Pharmacological Research* **64:**4-10.

Stedman C, Robertson G, Coulter S and Liddle C (2004) Feed-forward regulation of bile acid detoxification by CYP3A4: Studies in humanized transgenic mice. *Journal of Biological Chemistry* **279:**11336-11343.

Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P and Negishi M (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *Journal of Biological Chemistry* **274:**6043-6046.

Sueyoshi T, Moore R, Sugatani J, Matsumura Y and Negishi M (2008) PPP1R16A, the membrane subunit of protein phosphatase 1β, signals nuclear translocation of the nuclear receptor constitutive active/androstane receptor. *Molecular Pharmacology* **73:**1113-1121.

Suino K, Peng L, Reynolds R, Li Y, Cha JY, Repa JJ, Kliewer SA and Xu HE (2004) The nuclear xenobiotic receptor CAR: Structural determinants of constitutive activation and heterodimerization. *Molecular Cell* **16:**893-905.

Tanii H, Shitara Y and Horie T (2011) Population pharmacokinetic analysis of letrozole in Japanese postmenopausal women. *European Journal of Clinical Pharmacology* **67:**1017-1025.

Tao X, Piao H, Canney DJ, Borenstein MR and Nnane IP (2007) Biotransformation of letrozole in rat liver microsomes: Effects of gender and tamoxifen. *Journal of Pharmaceutical and Biomedical Analysis* **43:**1078-1085.

Tarirai C, Viljoen AM and Hamman JH (2010) Herb-drug pharmacokinetic interactions reviewed. *Expert Opinion on Drug Metabolism and Toxicology* **6:**1515-1538.

Teng S and Piquette-Miller M (2008) Regulation of transporters by nuclear hormone receptors: Implications during inflammation. *Molecular Pharmaceutics* **5:**67-76.

Thum T, Erpenbeck VJ, Moeller J, Hohlfeld JM, Krug N and Borlak J (2006) Expression of xenobiotic metabolizing enzymes in different lung compartments of smokers and nonsmokers. *Environmental Health Perspectives* **114:**1655-1661.

Tien ES and Negishi M (2006) Nuclear receptors CAR and PXR in the regulation of hepatic metabolism. *Xenobiotica* **36:**1152-1163.

Timsit YE and Negishi M (2007) CAR and PXR: The xenobiotic-sensing receptors. *Steroids* **72:**231-246.

Tirona RG and Bailey DG (2006) Herbal product-drug interactions mediated by induction. *British Journal of Clinical Pharmacology* **61:**677-681.

Tirona RG, Leake BF, Podust LM and Kim RB (2004) Identification of Amino Acids in Rat Pregnane X Receptor that Determine Species-Specific Activation. *Molecular Pharmacology* **65:**36-44.

Tirona RG, Lee W, Leake BF, Lan LB, Brimer Cline C, Lamba V, Parviz F, Duncan SA, Inoue Y, Gonzalez FJ, Schuetz EG and Kim RB (2003) The orphan nuclear receptor HNF4α determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nature Medicine* **9:**220-224.

Tolson AH, Li H, Eddington ND and Wang H (2009) Methadone induces the expression of hepatic drug-metabolizing enzymes through the activation of pregnane X receptor and constitutive androstane receptor. *Drug Metabolism and Disposition* **37:**1887-1894.

Tompkins LM and Wallace AD (2007) Mechanisms of cytochrome P450 induction. *Journal of Biochemical and Molecular Toxicology* **21:**176-181.

Toutain PL and Bousquet-Mélou A (2004a) Bioavailability and its assessment. *Journal of Veterinary Pharmacology and Therapeutics* **27:**455-466.

Toutain PL and Bousquet-Mélou A (2004b) Plasma clearance. *Journal of Veterinary Pharmacology and Therapeutics* **27:**415-425.

Toutain PL and Bousquet-Mélou A (2004c) Volumes of distribution. *Journal of Veterinary Pharmacology and Therapeutics* **27:**441-453.

Tredway D, Schertz JC, Bock D, Hemsey G and Diamond MP (2011) Anastrozole single-dose protocol in women with oligo- or anovulatory infertility: Results of a randomized phase II dose-response study. *Fertility and Sterility* **95:**1725-1729.e1728.

Tredway DR, Buraglio M, Hemsey G and Denton G (2004) A phase I study of the pharmacokinetics, pharmacodynamics, and safety of single- and multiple-dose anastrozole in healthy, premenopausal female volunteers. *Fertility and Sterility* **82:**1587-1593.

Tredway DR and Schertz JC (2011) Anastrozole versus clomiphene citrate: Which is better for ovulation induction? *Fertility and Sterility* **95:**1549-1551.

Turpeinen M, Tolonen A, Chesne C, Guillouzo A, Uusitalo J and Pelkonen O (2009) Functional expression, inhibition and induction of CYP enzymes in HepaRG cells. *Toxicology in Vitro* **23:**748-753.

Urquhart BL, Tirona RG and Kim RB (2007) Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: Implications for interindividual variability in response to drugs. *Journal of Clinical Pharmacology* **47:**566-578.

van Herwaarden AE, van Waterschoot RAB and Schinkel AH (2009) How important is intestinal cytochrome P450 3A metabolism? *Trends in Pharmacological Sciences* **30:**223-227.

Van Herwaarden AE, Wagenaar E, Van Der Kruijssen CMM, Van Waterschoot RAB, Smit JW, Song JY, Van Der Valk MA, Van Tellingen O, Van Der Hoorn JWA, Rosing H, Beijnen JH and Schinkel AH (2007) Knockout of cytochrome P450 3A yields new mouse models for understanding xenobiotic metabolism. *Journal of Clinical Investigation* **117:**3583-3592.

Van Waterschoot RAB, Rooswinkel RW, Sparidans RW, Van Herwaarden AE, Beijnen JH and Schinkel AH (2009) Inhibition and stimulation of intestinal and hepatic CYP3A activity: Studies in humanized CYP3A4 transgenic mice using triazolam. *Drug Metabolism and Disposition* **37:**2305-2313.

Videau O, Delaforge M, Levi M, Thévenot E, Gal O, Becquemont L, Beaune P and Bénech H (2010) Biochemical and analytical development of the CIME cocktail for drug fate assessment in humans. *Rapid Communications in Mass Spectrometry* **24:**2407-2419.

Vignati LA, Bogni A, Grossi P and Monshouwer M (2004) A human and mouse pregnane X receptor reporter gene assay in combination with cytotoxicity measurements as a tool to evaluate species-specific CYP3A induction. *Toxicology* **199:**23-33.

Wada T, Gao J and Xie W (2009) PXR and CAR in energy metabolism. *Trends in Endocrinology and Metabolism* **20:**273-279.

Wagner M, Halilbasic E, Marschall HU, Zollner G, Fickert P, Langner C, Zatloukal K, Denk H and Trauner M (2005) CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. *Hepatology* **42:**420-430.

Wang CY, Li CW, Chen JD and Welsh WJ (2006) Structural model reveals key interactions in the assembly of the pregnane X receptor/corepressor complex. *Molecular Pharmacology* **69:**1513-1517.

Wang D, Li L, Fuhrman J, Ferguson S and Wang H (2011) The role of constitutive androstane receptor in oxazaphosphorine-mediated induction of drug-metabolizing enzymes in human hepatocytes. *Pharmaceutical Research* **28:**2034-2044.

Wang H, Faucette S, Sueyoshi T, Moore R, Ferguson S, Negishi M and LeCluyse EL (2003) A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. *Journal of Biological Chemistry* **278:**14146-14152.

Wang H and Negishi M (2003) Transcriptional regulation of cytochrome P450 2B genes by nuclear receptors. *Current Drug Metabolism* **4:**515-525.

Wang H and Tompkins LM (2008) CYP2B6: New insights into a historically overlooked cytochrome P450 isozyme. *Current Drug Metabolism* **9:**598-610.

Wang X, Sykes DB and Miller DS (2010) Constitutive androstane receptor-mediated up-regulation of ATP-driven xenobiotic efflux transporters at the blood-brain barrier. *Molecular Pharmacology* **78:**376-383.

Washio I, Maeda M, Sugiura C, Shiga R, Yoshida M, Nonen S, Fujio Y and Azuma J (2011) Cigarette smoke extract induces CYP2B6 through constitutive androstane receptor in hepatocytes. *Drug Metabolism and Disposition* **39:**1-3.

Watkins RE, Davis-Searles PR, Lambert MH and Redinbo MR (2003) Coactivator binding promotes the specific interaction between ligand and the pregnane X receptor. *Journal of Molecular Biology* **331:**815-828.

Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP, Willson TM, Kliewer SA and Redinbo MR (2001) The human nuclear xenobiotic receptor PXR: Structural determinants of directed promiscuity. *Science* **292:**2329-2333.

Waxman DJ and Holloway MG (2009) Sex differences in the expression of hepatic drug metabolizing enzymes. *Molecular Pharmacology* **76:**215-228.

Waxman DJ, Pampori NA, Ram PA, Agrawal AK and Shapiro BH (1991) Interpulse interval in circulating growth hormone patterns regulates sexually dimorphic expression of hepatic cytochrome P450. *Proceedings of the National Academy of Sciences of the United States of America* **88:**6868-6872.

Wei P, Zhang J, Dowhan DH, Han Y and Moore DD (2002) Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. *Pharmacogenomics Journal* **2:**117-126.

Wei P, Zhang J, Egan-Haftey M, Liang S and Moore DD (2000) The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. *Nature* **407:**920-923.

Wempe MF, Buchanan CM, Buchanan NL, Edgar KJ, Hanley GA, Ramsey MG, Skotty JS and Rice PJ (2007) Pharmacokinetics of letrozole in male and female rats: Influence of complexation with hydroxybutenyl-β-cyclodextrin. *Journal of Pharmacy and Pharmacology* **59:**795-802.

Werneke U, Earl J, Seydel C, Horn O, Crichton P and Fannon D (2004) Potential health risks of complementary alternative medicines in cancer patients. *British Journal of Cancer* **90:**408-413.

Williamson EM (2003) Drug Interactions between Herbal and Prescription Medicines. *Drug Safety* **26:**1075-1092.

Wiwi CA, Gupte M and Waxman DJ (2004) Sexually dimorphic P450 gene expression in liver-specific hepatocyte nuclear factor 4α-deficient mice. *Molecular Endocrinology* **18:**1975-1987.

Won CS, Oberlies NH and Paine MF (2012) Mechanisms underlying food-drug interactions: Inhibition of intestinal metabolism and transport. *Pharmacology and Therapeutics* **136:**186-201.

Xia J and Kemper B (2007) Subcellular trafficking signals of constitutive androstane receptor: Evidence for a nuclear export signal in the DNA-binding domain. *Drug Metabolism and Disposition* **35:**1489-1494.

Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, Neuschwander-Tetri BA, Brunt EM, Guzelian PS and Evans RM (2000) Humanized xenobiotic response in mice expressing nuclear receptor SXR. *Nature* **406:**435-439.

Xie W, Yeuh MF, Radominska-Pandya A, Saini SPS, Negishi Y, Bottroff BS, Cabrera GY, Tukey RH and Evans RM (2003) Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. *Proceedings of the National Academy of Sciences of the United States of America* **100:**4150-4155.

Xie YB, Nedumaran B and Choi HS (2009) Molecular characterization of SMILE as a novel corepressor of nuclear receptors. *Nucleic Acids Research* **37:**4100-4115.

Xu J, Kulkarni SR, Li Y and Slitt AL (2012) UDP-glucuronosyltransferase expression in mouse liver is increased in obesity- and fasting-induced steatosis. *Drug Metabolism and Disposition* **40:**259-266.

Xu RX, Lambert MH, Wisely BB, Warren EN, Weinert EE, Waitt GM, Williams JD, Collins JL, Moore LB, Willson TM and Moore JT (2004) A structural basis for constitutive activity in the human CAR/RXRα heterodimer. *Molecular Cell* **16:**919-928.

Yacovino LL and Aleksunes LM (2012) Renal efflux transporter expression in pregnant mice with Type I diabetes. *Toxicology Letters* **211:**304-311.

Yamamoto Y, Kawamoto T and Negishi M (2003) The role of the nuclear receptor CAR as a coordinate regulator of hepatic gene expression in defense against chemical toxicity. *Archives of Biochemistry and Biophysics* **409:**207-211.

Yao R, Yasuoka A, Kamei A, Kitagawa Y, Tateishi N, Tsuruoka N, Kiso Y, Sueyoshi T, Negishi M, Misaka T and Abe K (2010) Dietary flavonoids activate the constitutive androstane receptor (CAR). *Journal of Agricultural and Food Chemistry* **58:**2168-2173.

Yasuda K, Ranade A, Venkataramanan R, Strom S, Chupka J, Ekins S, Schuetz E and Bachmann K (2008) A Comprehensive in Vitro and in Silico Analysis of Antibiotics That Activate Pregnane X Receptor and Induce CYP3A4 in Liver and Intestine. *Drug Metabolism and Disposition* **36:**1689-1697.

Yates RA, Dowsett M, Fisher GV, Selen A and Wyld PJ (1996) Arimidex (ZD1033): A selective, potent inhibitor of aromatase in post-menopausal female volunteers. *British Journal of Cancer* **73:**543-548.

Yoshinari K, Kobayashi K, Moore R, Kawamoto T and Negishi M (2003) Identification of the nuclear receptor CAR:HSP90 complex in mouse liver and

recruitment of protein phosphatase 2A in response to phenobarbital. *FEBS Letters* **548:**17-20.

Yoshinari K, Yoda N, Toriyabe T and Yamazoe Y (2010) Constitutive androstane receptor transcriptionally activates human CYP1A1 and CYP1A2 genes through a common regulatory element in the 5'-flanking region. *Biochemical Pharmacology* **79:**261-269.

Young E, Miele L, Tucker KB, Huang M, Wells J and Gu JW (2010) SU11248, a selective tyrosine kinases inhibitor suppresses breast tumor angiogenesis and growth via targeting both tumor vasculature and breast cancer cells. *Cancer Biology and Therapy* **10:**703-711.

Yuan J, Wang PQ, Ge SR, An FR and Shi AG (2001) Pharmacokinetics of anastrozole in Chinese male volunteers. *Acta Pharmacologica Sinica* **22:**573-576.

Yueh MF, Kawahara M and Raucy J (2005) High volume bioassays to assess CYP3A4-mediated drug interactions: Induction and inhibition in a single cell line. *Drug Metabolism and Disposition* **33:**38-48.

Yueh MF, Mellon PL and Tukey RH (2011) Inhibition of human UGT2B7 gene expression in transgenic mice by the constitutive androstane receptor. *Molecular Pharmacology* **79:**1053-1060.

Zaman K, Thürlimann B, Huober J, Schönenberger A, Pagani O, Lüthi J, Simcock M, Giobbie-Hurder A, Berthod G, Genton C, Brauchli P and Aebi S (2012) Bone mineral density in breast cancer patients treated with adjuvant letrozole, tamoxifen, or sequences of letrozole and tamoxifen in the BIG 1-98 study (SAKK 21/07). *Annals of Oncology* **23:**1474-1481.

Zelko I, Sueyoshi T, Kawamoto T, Moore R and Negishi M (2001) The peptide near the C terminus regulates receptor CAR nuclear translocation induced by xenochemicals in mouse liver. *Molecular and Cellular Biology* **21:**2838-2846.

Zhang J, Huang W, Chua SS, Wei P and Moore DD (2002) Modulation of acetaminophen-induced hepatotoxicity by the xenobiotic receptor CAR. *Science* **298:**422-424.

Zhang L, Reynolds KS, Zhao P and Huang SM (2010) Drug interactions evaluation: an integrated part of risk assessment of therapeutics. *Toxicology and applied pharmacology* **243:**134-145.

Zhang W, Purchio AF, Chen K, Wu J, Lu L, Coffee R, Contag PR and West DB (2003) A transgenic mouse model with a luciferase reporter for studying in vivo transcriptional regulation of the human CYP3A4 gene. *Drug Metabolism and Disposition* **31:**1054-1064.

Zhou J, Zhang J and Xie W (2005) Xenobiotic nuclear receptor-mediated regulation of UDP-glucuronosyltransferases. *Current Drug Metabolism* **6:**289-298.

Zhou SF and Lai X (2008) An update on clinical drug interactions with the herbal antidepressant St. John's wort. *Current Drug Metabolism* **9:**394-409.

Zidan J, Chetver L, Hussein O and Zucker M (2010) Effect of letrozole on plasma lipids, triglycerides, and Estradiol in postmenopausal women with metastatic breast cancer. *Oncologist* **15:**1159-1163.

Zimmermann C, van Waterschoot RAB, Harmsen S, Maier A, Gutmann H and Schinkel AH (2009) PXR-mediated induction of human CYP3A4 and mouse Cyp3a11 by the glucocorticoid budesonide. *European Journal of Pharmaceutical Sciences* **36:**565-571.

APPENDIX A

rCYP2B6-LACZ RAW DATA

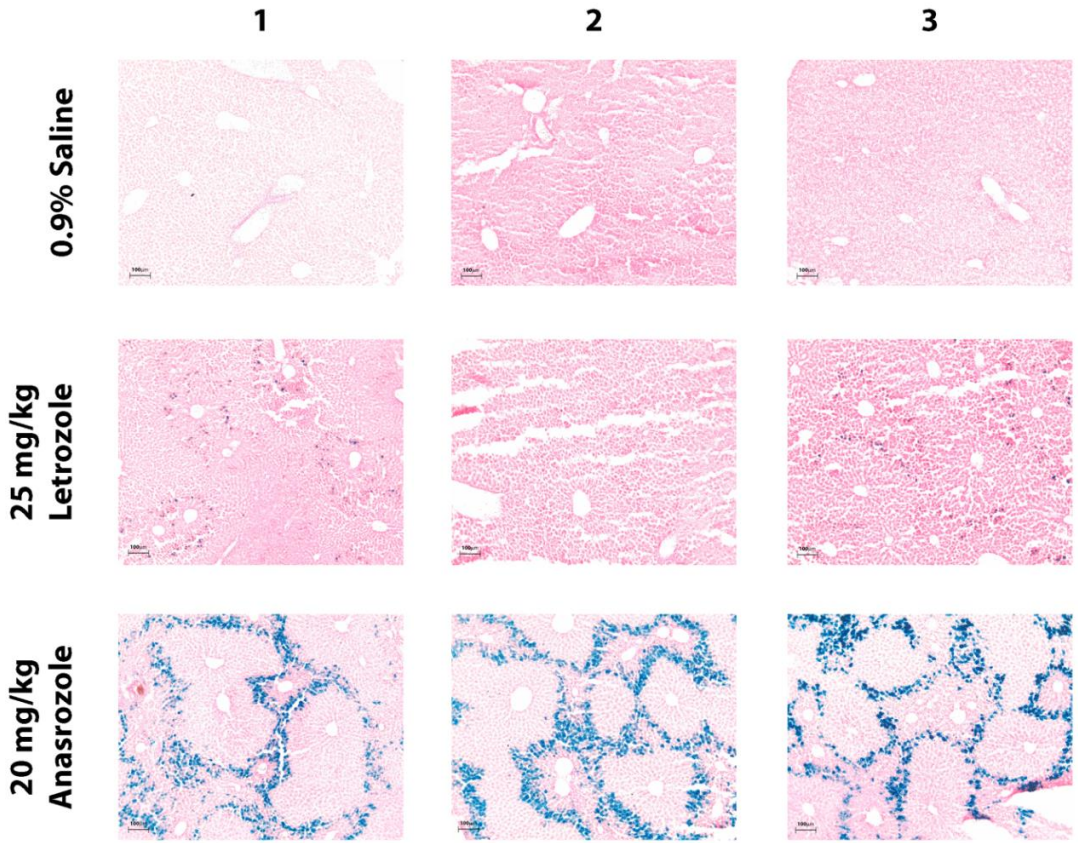


Figure A.I: LacZ staining following 3 day drug treatment of female CYP2B6-LacZ (mPXR/mCAR) reporter mice (10x magnification)

Female CYP2B6-LacZ (mPXR/mCAR) reporter mice (n=3 per group) were treated p.o. daily for 3 days with 0.9% Saline, 25 mg/kg Letrozole or 20 mg/kg Anastrozole. Livers were harvested into 1% PFA (w/v), cryofixed and sectioned before staining for nuclear fast red, followed by LacZ. Photos were taken at 10x magnification. Scales indicate 100mm. Numbers refer to treatment subject.

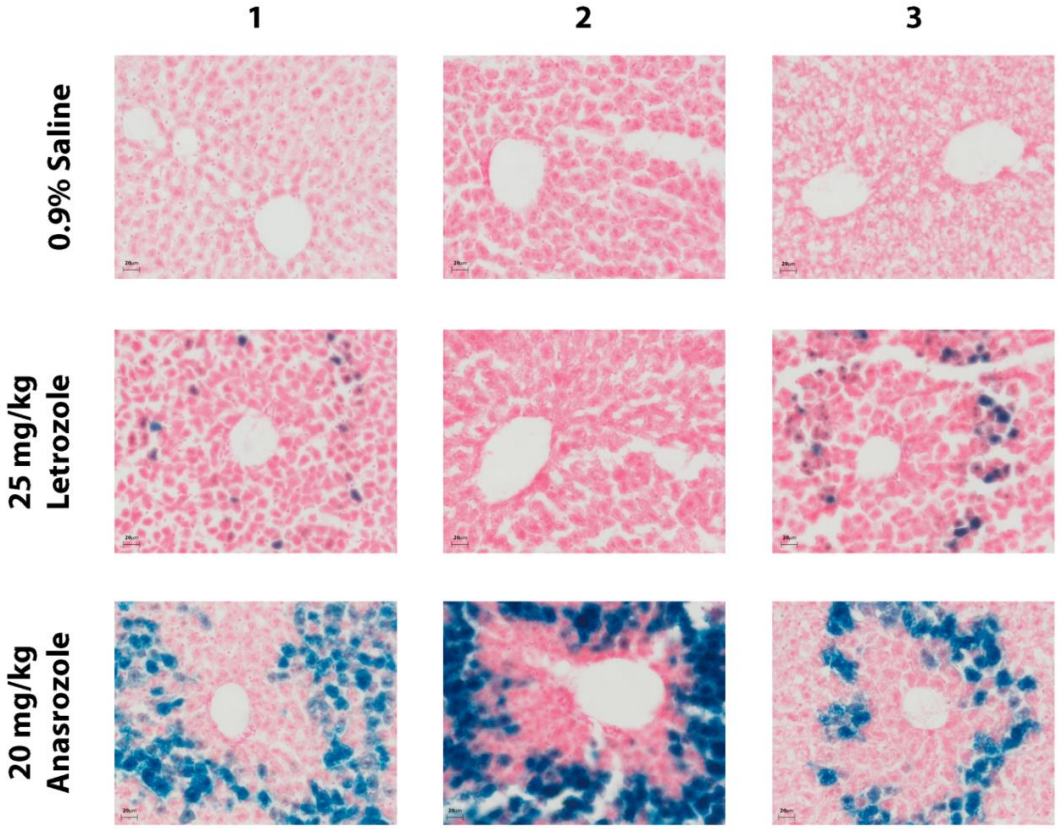


Figure A.II: LacZ staining following 3 day drug treatment of female CYP2B6-LacZ (mPXR/mCAR) reporter mice (40x magnification)

Female CYP2B6-LacZ (mPXR/mCAR) reporter mice (n=3 per group) were treated p.o. daily for 3 days with 0.9% Saline, 25 mg/kg Letrozole or 20 mg/kg Anastrozole. Livers were harvested into 1% PFA (w/v), cryofixed and sectioned before staining for nuclear fast red, followed by LacZ. Photos were taken at 40x magnification. Scales indicate 20mm. Numbers refer to treatment subject.

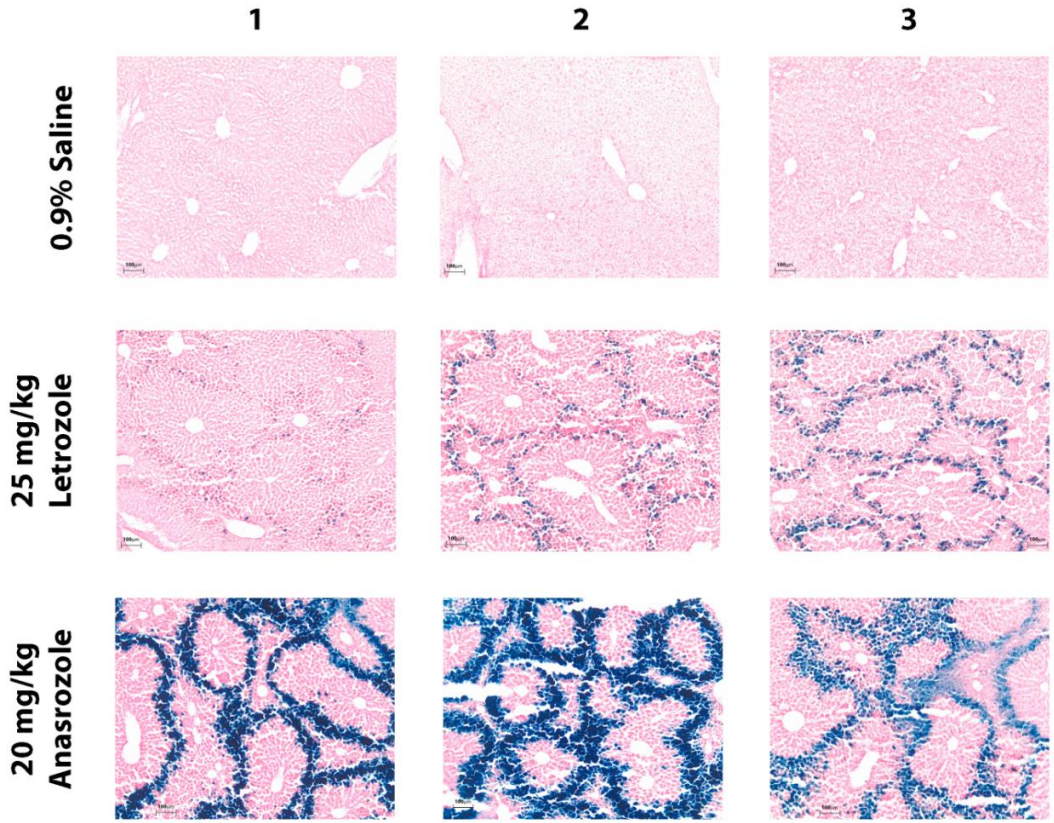


Figure A.III: LacZ staining following 3 day drug treatment of male CYP2B6-LacZ (mPXR/mCAR) reporter mice (10x magnification)

Male CYP2B6-LacZ (mPXR/mCAR) reporter mice (n=3 per group) were treated p.o. daily for 3 days with 0.9% Saline, 25 mg/kg Letrozole or 20 mg/kg Anastrozole. Livers were harvested into 1% PFA (w/v), cryofixed and sectioned before staining for nuclear fast red, followed by LacZ. Photos were taken at 10x magnification. Scales indicate 100mm. Numbers refer to treatment subject.

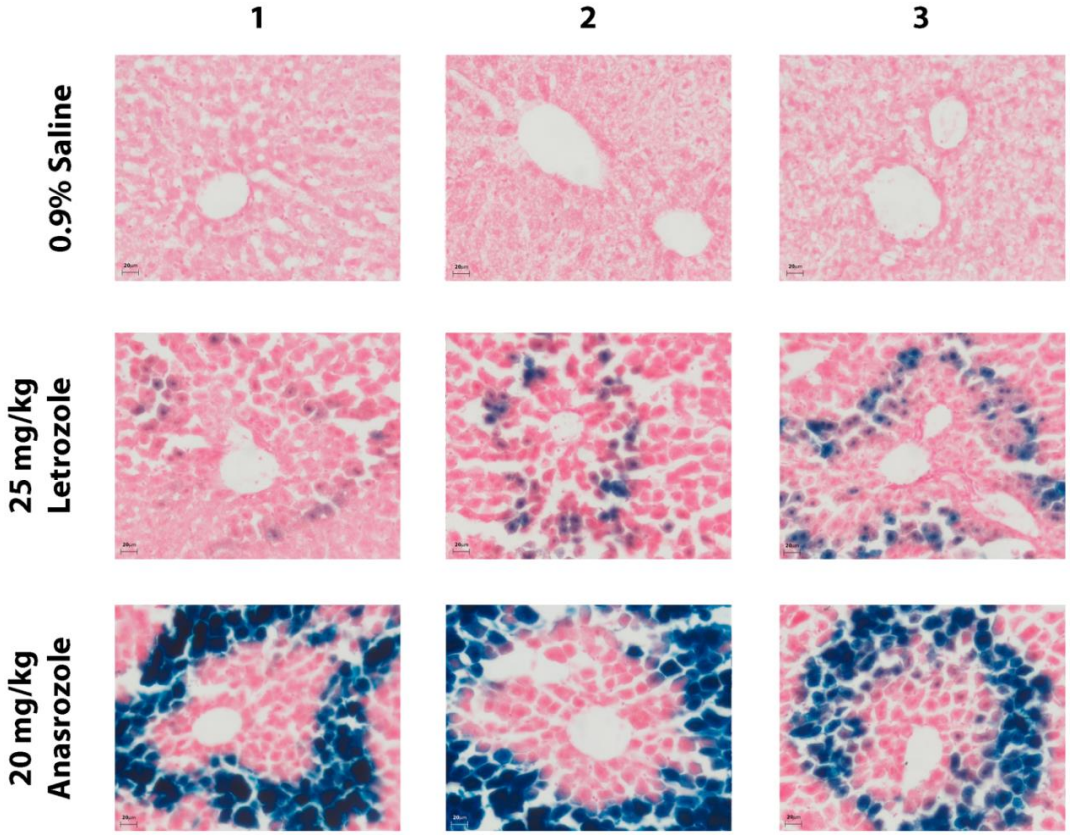


Figure A.IV: LacZ staining following 3 day drug treatment of male CYP2B6-LacZ (mPXR/mCAR) reporter mice (40x magnification)

Male CYP2B6-LacZ (mPXR/mCAR) reporter mice (n=3 per group) were treated p.o. daily for 3 days with 0.9% Saline, 25 mg/kg Letrozole or 20 mg/kg Anastrozole. Livers were harvested into 1% PFA (w/v), cryofixed and sectioned before staining for nuclear fast red, followed by LacZ. Photos were taken at 40x magnification. Scales indicate 20mm. Numbers refer to treatment subject.

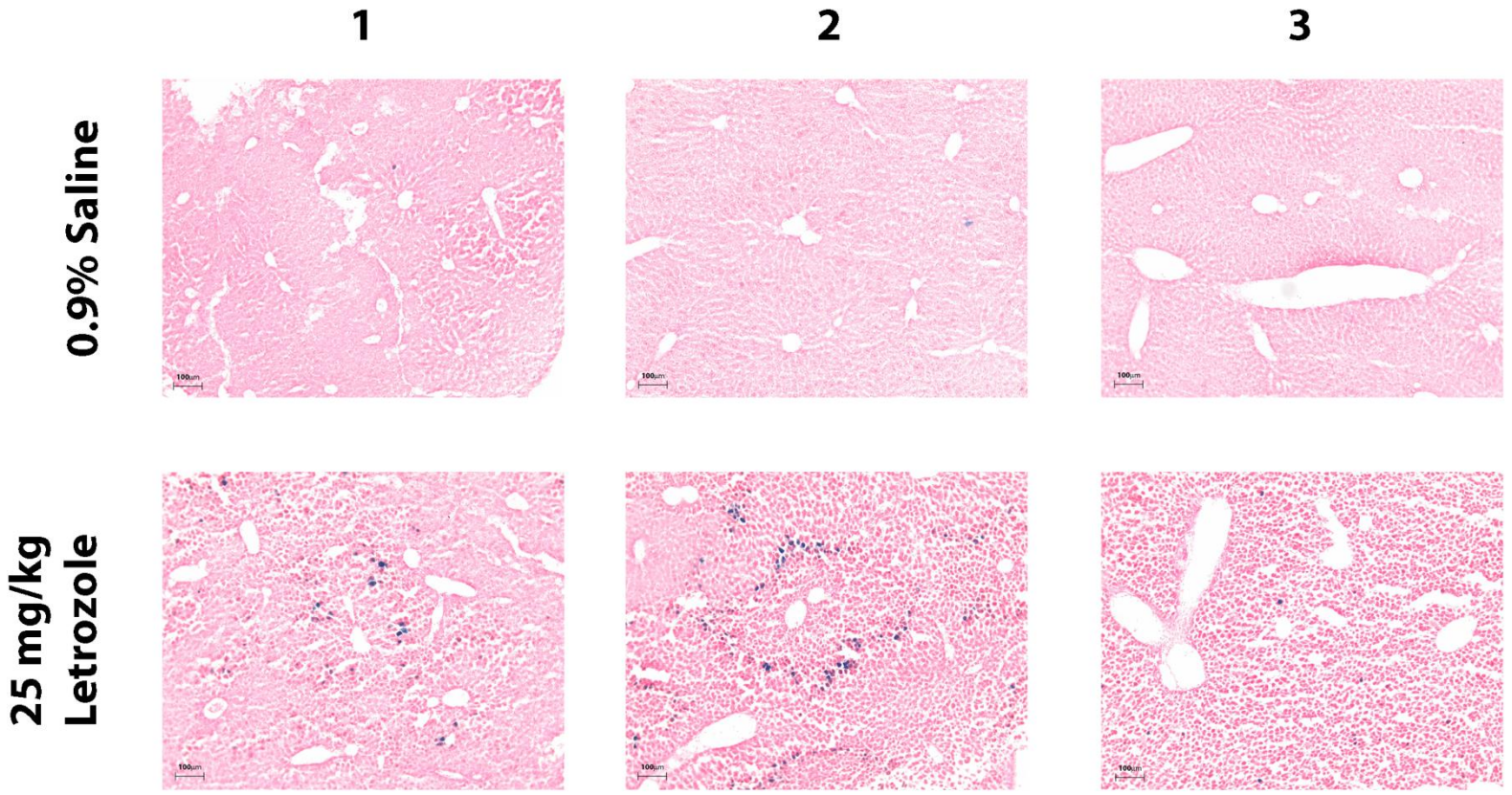


Figure A.V: LacZ staining following 3 day drug treatment of male CYP2B6-LacZ (hPXR/hCAR) reporter mice (10x magnification)

Male CYP2B6-LacZ (hPXR/hCAR) reporter mice (n=3 per group) were treated p.o. daily for 3 days with 0.9% Saline or 25 mg/kg Letrozole. Livers were harvested into 1% PFA (w/v), cryofixed and sectioned before staining for nuclear fast red, followed by LacZ. Photos were taken at 10x magnification. Scales indicate 100mm. Numbers refer to treatment subject.

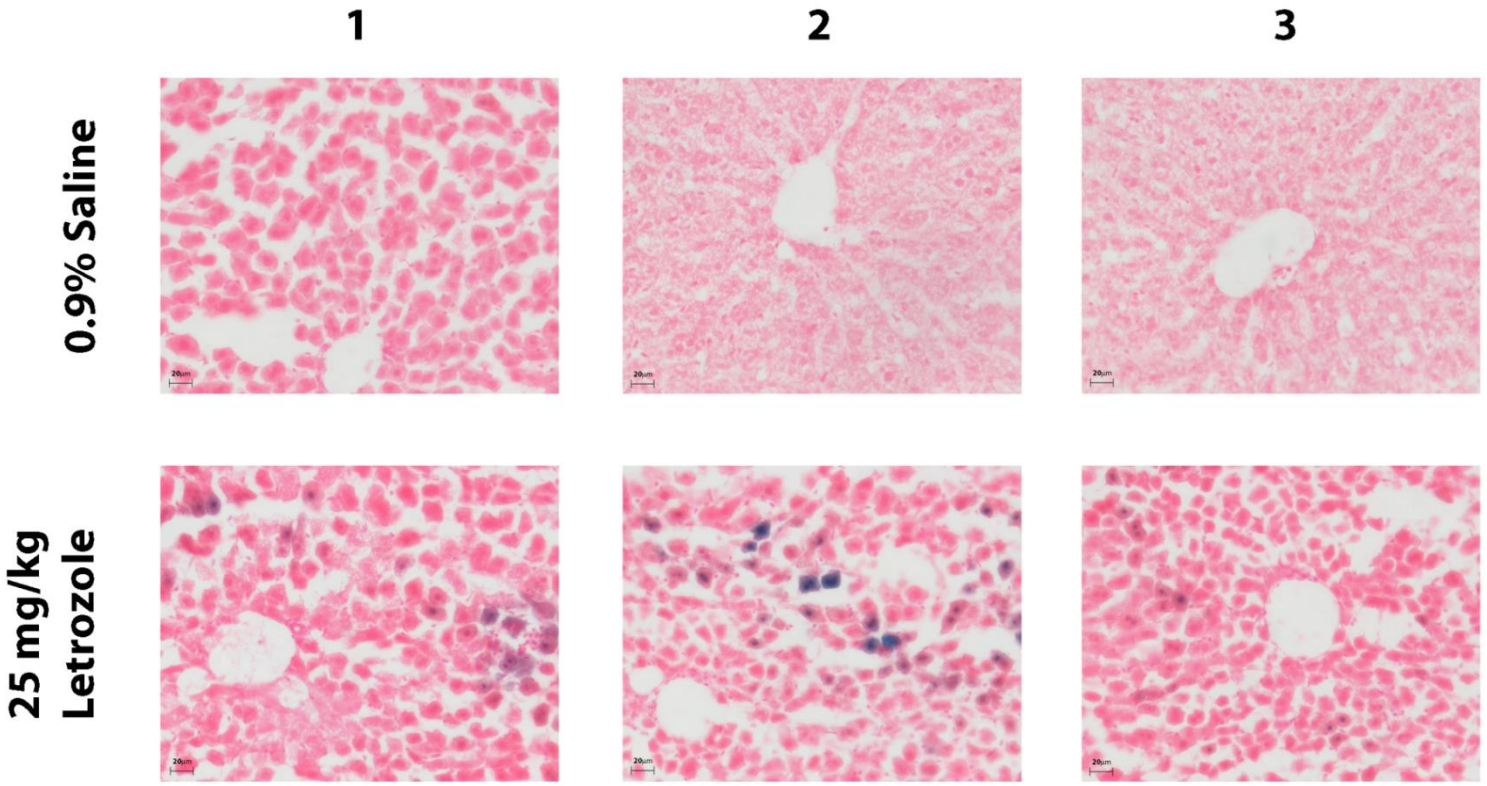


Figure A.VI: LacZ staining following 3 day drug treatment of male CYP2B6-LacZ (hPXR/hCAR) reporter mice (40x magnification)

Male CYP2B6-LacZ (hPXR/hCAR) reporter mice (n=3 per group) were treated p.o. daily for 3 days with 0.9% Saline or 25 mg/kg Letrozole. Livers were harvested into 1% PFA (w/v), cryofixed and sectioned before staining for nuclear fast red, followed by LacZ. Photos were taken at 40x magnification. Scales indicate 20mm. Numbers refer to treatment subject.

APPENDIX B

EO771 INITIAL ANALYSIS

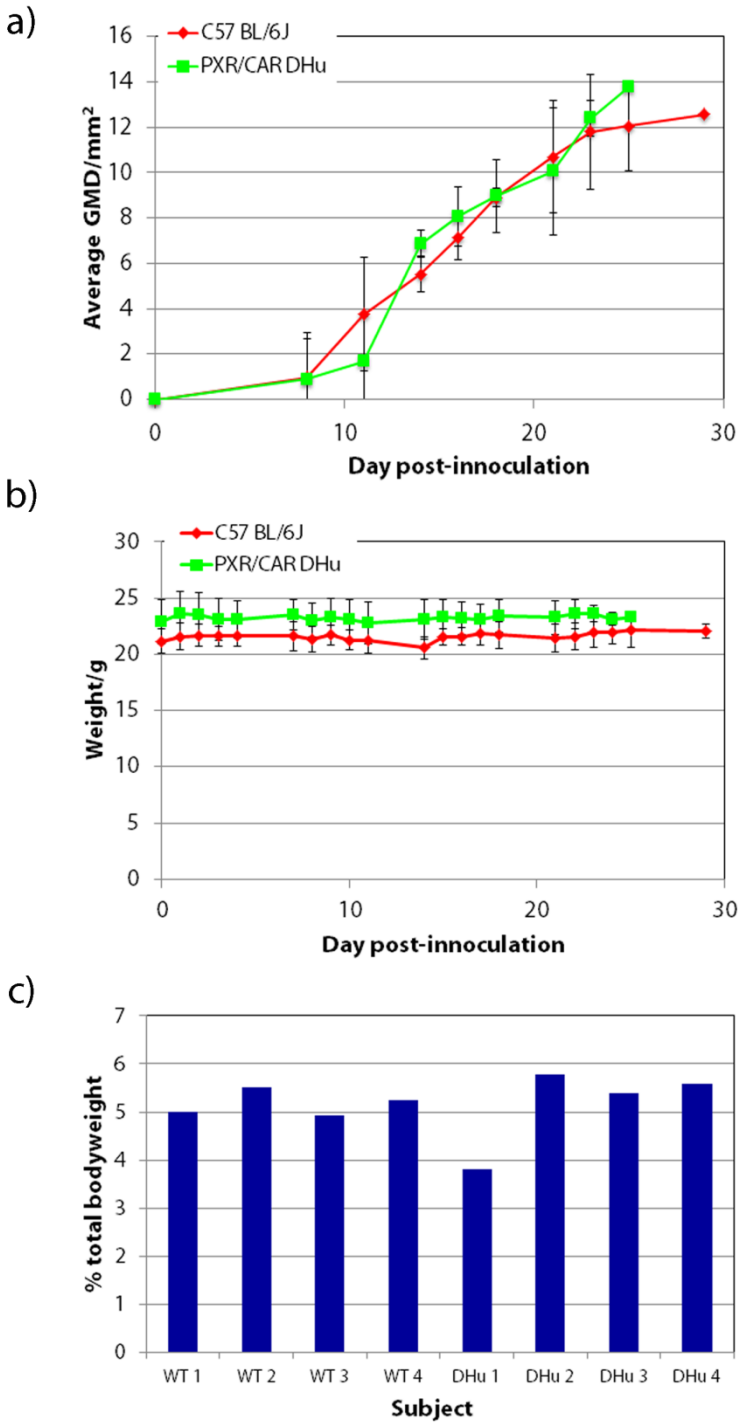


Figure B.I: E0771 syngeneic mouse model pilot growth data

C57BL/6J wild type (n=5) and PXR/CAR DHu transgenic (n=4) mouse models were innoculated with 2.5 x 106 E0771 metastatic breast adenocarcinoma cells into their right flanks under Euthatal anaesthesia (Day 0). Geometric mean dimension was measured twice weekly, with mice being euthanised when tumours reached 12.5mm2 (a). Body weight was measured daily (b) and liver weight noted following harvest (c). Tumours did not form in one C57BL/6Jwild type mouse. This has been excluded from these data.

APPENDIX C

RECENT PUBLICATION

8 Transcriptional Regulation of Cytochrome P450 Genes

EMILY A. FRASER, COLIN J. HENDERSON, and C. ROLAND WOLF
Division of Cancer Research, University of Dundee, Medical Research Institute, Ninewells Hospital & Medical School, Dundee, UK

8.1 INTRODUCTION

Our mechanistic understanding of the transcriptional regulation of cytochrome P450 (P450) expression has increased significantly in recent years, with the discovery and characterization of "orphan" nuclear receptors such as the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR). The role of these receptors as mediators of the inductive effects of xenobiotics continues to be the subject of intensive investigation and has added a further layer of complexity to an already burgeoning area of research.

It has become clear that significant variability exists at almost every level of the processes that contribute to the ultimate functionality of P450 proteins, and transcriptional regulation is no exception. Expression of these proteins is controlled by transcription factors that not only vary in their expression level and the degree to which they bind and respond to different classes of ligands but are also themselves subject to alternative splicing, producing variant receptor forms with different spatiotemporal expression and functionality. It is clear that only by gaining a detailed understanding of these receptors will we have a greater appreciation of the intricacies of transcriptional regulation of P450s.

This chapter concentrates on those transcription factors involved in xenobiotic-induced expression, giving an overview of regulatory interactions and raising issues that have a bearing on transcriptional regulation, such as polymorphisms, splice variants, and species specificity. However, it is important to note that other transcription

Encyclopedia of Drug Metabolism and Interactions, 6-Volume Set, First Edition.
Edited by Alexander V. Lyubimov.

factors that are not covered in this review - including the peroxisome proliferator activated receptors α and γ, the vitamin D receptor, and the oestrogen receptors α and β - are also involved in regulation of endogenous and basal cytochrom P450 expression.

8.2 TRANSCRIPTIONAL REGULATION OF CYTOCHROME P450s

8.2.1 Pregnane X Receptor (PXR)

The pregnane X receptor (PXR, also known as SXR, PAR, and NR1I2) is the most recently identified of the three main regulators of xenobiotic-induced CYP expression, with the mouse ortholog having been first identified by Kliewer *et al*. in 1998, closely followed by the identification of the human form by Blumberg *et al*. [1,2]. A ligand-activated orphan nuclear receptor was named the pregnane X receptor because of its activation by pregnenolone derivatives. Acting as a xenosensor, PXR is one of the key controllers of drug metabolism, in particular, for the expression of the CYP3A enzyme isoforms. In addition to the CYP3A enzymes, PXR, bound as a heterodimer to its partner, the retinoid X receptor (RXR), regulates a wide variety of P450s, as well as phase II enzymes and phase III drug transporters (Table 8.1). PXR is also promiscuous, being able to accommodate and be activated by a wide range of different ligands, both endogenous and exogenous (Table 8.2). This protein is expressed in many tissues, including those of heart, colon, stomach, and certain brain regions, although it is primarily expressed in the liver and small intestine [3]. Several splice variants have also been identified, which affect PXR-mediated transcriptional regulation (Section 8.3.3).

Flexibility in the ligand-binding domain (LBD) of PXR is the reason for its wide substrate specificity, with the binding pocket able to expand from its resting 1150 $Å^3$ to more than 1600 $Å^3$ when ligand bound. Large molecules, such as the macrolide antibiotic rifampicin (RIF), can therefore activate the receptor using an induced-fit

TABLE 8.1 Examples of Human Cytochrome P450s Regulated by Human PXR, CAR, and AHR

Gene	PXR	CAR	AHR	References
CYP1A1	—	—	↑[a]	[4][a]
CYP1A2	↑	↑[b]	↑[a]	[5][b] and [4][a]
CYP1B1	↑	↔	↑	[6,7]
CYP2A6	↑[b]	↑	—	[8][b]
CYP2B6	↑	↑	↔	—
CYP2C8	↑	↑	—	[9]
CYP2C9	↑	↔	↔[b]	[10] and [4][b]
CYP2C19	↑	↑	—	—
CYP3A4	↑	↑	↔	—
CYP7A1	↓[b]	↓[a]	↔[c]	[11],[b] [12],[a] and [4][c]
CYP8B1	↓[b]	↓[p]	—	[11][b] and [13]
CYP24A1	↑	↑	—	[14]

Bold arrows indicate strong induction.

↑, induced; ↓, repressed; ↔, no change/basal expression; [p], putative interaction in humans. a, b, or c on each line refers change in nuclear receptor expression to the corresponding citation.

Source: Unless otherwise stated, data was extracted from Ref. 15.

TABLE 8.2 Example Modulators of Human PXR, CAR, and AHR

Class	Compound	PXR	CAR	AHR	References
Classical	Rifampicin	++			PXR/CAR: [7,15–21] AHR: [6,22]
	CITCO		++		
	TCDD			++	
	3-Methylcholanthrene			++	
Drug	Omeprazole	+		+	
	Thiabendazole			+	
	Nicotine			+	
	Caffeine			+	
	Paclitaxel	+			
	Methadone	+	+		
	Clotrimazole		−		
	Ketoconazole	−	−		
	17α-Ethinylestradiol	+			
	Mifepristone	+			
	SR12 813	+			
	Phenytoin	+			
	Primaquine	+			
	Spironolactone	+			
Herb	Hyperforin	++			
	Cryptotanshinone	+			
	Artemisinin	+	+		
	Ginkgo biloba	+	+		
	Schisandrin A-C	+			
Dietary	β-Carotene	+		+	
	Vitamin E	+			
	Flavonoids			±	
	Curcumin			+	
Endogenous	Androstanol		+		
	Corticosterone	+			
	17β-Estradiol	+	+		
	Vitamin K_2	+			
	Tryptophan metabolites			+	
	Bilirubin			+	
Environmental/ industrial	Benzyl butyl phthalate	+			
	Chlordane	+			
	Phthalic acid (DHEP)	+	+		
	Nonylphenol	+	+		
	Polychlorinated biphenyls	+			
	Toxaphene	+			
	Triclopyr	+			
	Benzo(*a*)pyrene			+	
	1-Methyl-1-phenylhydrazine			+	

Blank spaces indicate no data is available.

++, very strong agonist; +, agonist; –, antagonist (or inverse agonist, CAR only); ±, antagonist/agonist, dependent on cell context.

For further information see reviews [6,15–17].

mechanism, which allows molecules to adopt various binding orientations before selecting the optimal configuration [23–25]. The LBD is predominantly hydrophobic in character, with Met243, Ser247, Gln285, Trp299, His407, and Phe420 commonly interacting with ligands [26,27]. Site-directed mutagenesis studies have indicated that even small changes in the LBD can have significant effects on the ligand-induced activation of PXR [25,26]. Interspecies variability in this region therefore has significant effects on the ligand activation profile (Section 8.3.2).

Nuclear translocation is a controlling factor in PXR-mediated P450 induction. However, because of difficulties with investigating translocation *in vitro*, namely, when PXR is overexpressed it spontaneously translocates to the nucleus [28], data regarding this mechanism remains limited. It has been reported that when inactive, PXR is bound in a complex with cytoplasmic CAR retention protein (CCRP) and HSP90, which retains the receptor in the cytoplasm [29,30]. Although the mechanism underlying PXR dissociation from the complex and translocation to the nucleus has not yet been elucidated, it is likely that a mechanism similar to that governing CAR dissociation from the retention complex is involved (Section 8.2.2). Once released from the cytoplasmic retention complex, PXR nuclear localization signals (NLSs) are targeted by importin-α proteins for nuclear import [29].

Upon entering the nucleus, PXR heterodimerizes with its binding partner, the RXRα, before binding to response elements in the promoter and enhancer regions of target genes (Fig. 8.1). PXR heterodimers preferentially bind direct repeat sequences possessing the half site AG(G/T)TCA separated by three nucleotides (DR3) and everted repeats of the half site separated by six nucleotides (ER6), that is, AG(G/T)TCAnnnAG(G/T)TCA and TGA(A/C)CTnnnnnnAG(G/T)TCA, respectively [15,16]. However, they are also able to bind other recognition sites, such as DR4, DR5, and ER8 motifs, although with lower affinity [31], thus accounting for the cross talk between receptors, which is an important contributor to P450 regulation.

A key component of transcriptional activation by nuclear receptors is the recruitment of coactivators and corepressors to promoter regions, following DNA binding. Coactivators of PXR, CAR, and AHR (aryl hydrocarbon receptor) are given in Table 8.3. As an example, PXR is a binding partner for the p160/SRC (steroid receptor coactivator) coactivator family, which recruits histone acetyltransferase complexes, such as cAMP response element binding protein/p300 (CREB), thus providing access to DNA strands for RNA-polymerase-catalyzed transcription [25]. These coactivators possess three LXXLL motifs that bind to the AF-2 helix, interacting with two charged residues on the receptor surface (Lys259 and Glu427) to form a charge clamp [24]. Crystal structures of SR12813 in complex with PXR and SRC-1 indicate that in the presence of coactivator, the ligand will bind in a single orientation. The combination of ligand and SRC-1 binding stabilizes the protein structure to restrict the flexibility of the LBD once an active ligand conformation has been achieved [25]. In contrast, corepressors are thought to bind in the absence of ligand, or in the presence of antagonists, retaining the AF-2 helix in a nonactive conformation [16]. Corepressors of PXR include the nuclear receptor corepressor (NCoR) and the silencing mediator of retinoid and thyroid hormone receptor (SMRT). SMRT possesses both ID1 and ID2 interacting domains, which consist of a CoRNR box (I/LXXI/VI) and the motif LXXXIXXXI/L, respectively [32]. PXR binds to the ID2 motif preferentially over the ID1, with the key interacting residues being Lys259, Gly270, and Pro423 of the PXR LBD and Arg2347, Lys2348, and Leu2350 of SMRT [32]. Site-directed mutagenesis studies show that mutation

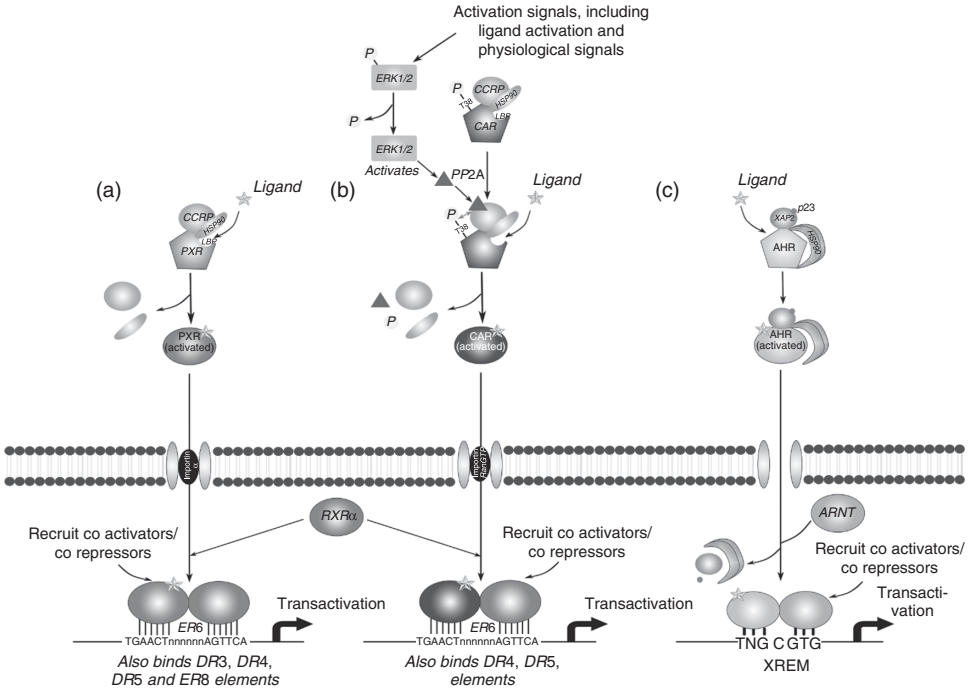


Figure 8.1 Activation mechanisms of (a) PXR, (b) CAR, and (c) AHR. (See color insert.)

TABLE 8.3 Coactivators and Corepressors Associated with PXR, CAR, and AHR

Coregulator Type	Coregulator	PXR	CAR	AHR	References
Coactivators	SRC-1	*	*	*	AHR: 33,34 PXR/CAR: 12, 15, 16, 35
	NCOA2 (GRIP1, SRC-2)	*	*	*	
	p/CIP (SRC-3)	*		*	
	P300			*	
	CBP			*	
	RIP 140	*		*	
	CARM1			*	
	PRMT1			*	
	PGC-1α	*	*		
	PBP	*	*		
	ASC-2		*		
	SMC-1		*		
Corepressors	ANKRA2			*	
	SMRT	*		*	
	NCoR	*	*		
	SHP	*			

[a]Corepression of AHR via the AHR repressor [36].

of charged residues within the PXR LBD (Arg410Asn, Asp205Ala, Glu321Ala, and Arg413Ala) increases the binding affinity for corepressors, indicating a core role for these residues in the maintenance of basal PXR activation [32]. Acting in concert, the recruitment of coregulators therefore controls the expression of target genes, such as P450s, by regulating access of RNA polymerases to the promoter regions.

A further aspect of PXR-mediated regulation of P450s is posttranslational modification of PXR by phosphorylation. This modification is known be critical to the correct function of other nuclear receptors, including CAR and AHR, but, until recently, had not been subjected to a systematic analysis with respect to PXR. Research from the Staudinger group has indicated that protein kinase A (PKA) interacts with PXR, but in a species-specific manner, synergizing with ligand activation in mice but repressing ligand-induced activation in rats and humans [37]. Protein kinase C (PKC) and CDK2 signaling has also been implicated. A recent mutagenesis study, in which 18 serine and threonine residues identified by either *in silico* site prediction or comparison with other receptors, has shed more light on the role of phosphorylation in PXR activity. These studies have shown that the phosphorylation state of certain key residues (Ser8, Thr57, Ser208, Ser305, Ser350, and Thr408) is involved in the control of heterodimerization, DNA binding, corepressor recruitment, and nuclear translocation [38]. The phosphorylation state of PXR therefore appears to be important in controlling gene expression. However, these observations require further studies to fully understand the role of phosphorylation in PXR function.

In summary, PXR is a key controller of P450 regulation, being associated with a wide variety of target genes. It also has an important role in many endogenous processes, including control of glucose homeostasis and the cholesterol and bile acid metabolism [15,16]. However, most importantly from a pharmaceutical perspective

is its role as the key regulator of the CYP3A enzymes, which are responsible for the metabolism of a significant proportion of currently used pharmaceuticals, and as a consequence involved in a significant number of drug–drug interactions [39]. As a key regulator of P450s, xenobiotics that interact with PXR could have a profound effect on their own metabolism as well as on the metabolism of other drugs given concomitantly. The promiscuity of PXR in terms of its ligand interactions and the wide range of genes it transactivates can result in severe drug–drug interactions [40–43]. As a consequence, PXR transactivation is investigated as a part of preclinical drug development programs. Regulation of this signaling pathway is still incompletely understood, although it is clear that it involves a complex interaction between coregulators, protein structure, and phosphorylation state.

8.2.2 Constitutive Androstane Receptor (CAR)

Initially identified in humans in 1994 as MB67 [44], and in the mouse in 1997 [45], the constitutive androstane receptor (CAR, NR1I3) is well established as a key regulator of P450 expression [16,39,46–57]. The genes targeted by CAR are numerous and varied, encompassing P450s (Table 8.1), phase II enzymes such as UDP-glucuronyltransferases, and phase III drug transporters, with CYP2B6 being prototypically induced (Cyp2b10 in mice, CYP2B1/2 in rat) [58,59]. Lacking a physiological ligand, this protein was also classed as an orphan nuclear receptor. However, as a nuclear receptor, CAR has some unusual properties, having both a different protein structure and displaying constitutive transcriptional activity [60].

As a consequence of the constitutive activity of CAR, molecules such as androstanol and androstenol have been referred to as *inverse agonists* because they actively repress constitutive CAR activity instead of antagonizing in the classical sense [61]. While not as promiscuous as PXR, CAR can be activated by numerous compounds (Table 8.2), but few operate by binding directly to CAR. Its constitutive basal activity means that it can transactivate genes without ligand binding, in response to externally acting signals that induce nuclear translocation [62]. This indirect activation pathway is a key mechanism in CAR-mediated transactivation, with phenobarbital (PB) induction of CYP2B being the classic example, and is suggested to be the major activation pathway for this molecule [63,64]. These phenomena are dependent on several key structural features of CAR and the increasingly understood mechanism of nuclear translocation, which are considered below.

Structurally, a protein consists of three domains: (i) a highly conserved N-terminal DNA-binding domain, (ii) a hinge region, and (iii) a variable C-terminal domain associated with ligand binding (AF-2 domain), dimerization with the CAR-binding partner (RXRα), and transcriptional activation [54]. The ligand-binding pocket (LBP) is significantly smaller than that of PXR at approximately 675 $Å^3$ in humans and is predominantly hydrophobic in nature. The conformation of the AF-2 domain within the LBP is thought to be vital to the constitutive activity of the protein, although it is not accessible for ligand binding, being protected by the side chains of Phe161 (also key in constitutive activity), Asn165, Phe234, and Tyr326, which are conserved across all mammalian orthologs of CAR [65,66]. It has also been suggested that disruption of the AF-2 conformation is responsible for switching CAR to its inactive form, such as is seen upon inverse agonist interaction [27].

Although CAR is described as constitutively active, it is unable to activate transcription of downstream genes in the absence of an external signal because of regulatory mechanisms controlling its translocation to the nucleus. In the absence of ligand or indirect activation, CAR is sequestered in the cytoplasm, bound to a complex consisting of CCRP and HSP90, along with other accessory proteins [67,68]. In response to direct or indirect ligand challenge, NLSs are activated, resulting in dissociation from this complex. Once the ligand is activated and released from the cytoplasmic retention complex, active CAR is translocated into the nucleus via an importin/Ran-GTP mediated process involving NLS binding to IPO13 [69].

The molecular trigger for, and underlying mechanism of, nuclear translocation is still unclear, although recent research has implied an essential role for cellular signaling pathways and phosphorylation status, especially in indirect control [28]. Although the role of phosphorylation state in CAR translocational control had been described previously, it was not until 2009 that the Thr38 amino acid was identified as the key residue in regulation of human constitutive androstane receptor (hCAR) translocation. Phosphorylation of Thr38 by PKC inactivates CAR by destabilizing the helix containing the C-terminal region of the first zinc finger, resulting in ablation of DNA binding [70]. However, on Thr38 dephosphorylation, the disrupted helix regains its stability, resulting in nuclear translocation and transactivation of downstream genes. It has been suggested that an essential process in PB-induced activation is the recruitment of protein phosphatase 2A (PP2A) to the retention protein complex in mouse hepatocytes, subsequently inducing translocation [71,72]. PP2A has therefore been suggested as a candidate for the dephosphorylation of this residue, acting in concert with other cofactors recruited to the CCRP/HSP90 complex. In addition, a recent report by Koike *et al*. identifies extracellular signal-regulated kinase (ERK) 1/2 as the endogenous signal controlling sequestration of CAR in the cytoplasm, with dephosphorylation of ERK 1/2 being sufficient to induce nuclear translocation [73]. AMP-activated protein kinase has also been suggested to influence CAR localization following PB treatment, although currently available data is conflicting [64,74–77]. Mutoh *et al*. suggest a model for both direct and indirect activators, in which PKC phosphorylates and PP2A dephosphorylates Thr38 to control nuclear translocation. The signal controlling the phosphorylation state of this residue appears to be extracellular signal-regulated kinase (ERK) 1/2 [70,73]. However, a membrane-bound regulatory subunit of protein phosphatase 1b, PPP1R16A, has recently been shown to interact with CAR, inducing translocation, which agrees with a report which localizes CAR at the cell surface in addition to the cytoplasm [78,79]. This suggests another layer of complexity in controlling nuclear translocation.

Once in the nucleus, CAR binds to RXRα in a head to tail arrangement and the resulting heterodimer binds to response elements in the promoter of target genes (Fig. 8.1). CAR will preferentially bind to three binding motifs: (i) DR4 (e.g., AGTTCAnnnnAGTTCA), (ii) DR5 (e.g., AGTTCAnnnnnAGTTCA), and (iii) ER8 (e.g., TGAACTnnnnnnnnAGTTCA). However, CAR can bind other motifs, such as ER5–ER10 types, but with lower affinity [15,80]. Unusually, CAR is also able to bind to DNA as a monomer, having a preference for DR4 motifs [80]. The optimal binding site for monomeric CAR was found to be AGAGTTCA. Binding to these motifs as a monomer is as strong as that of the T_3 thyroid hormone receptor, and the preference for monomeric binding is more marked in humans than in mice. However, if the 5′ nucleotide flanking sequences contain pyrimidine nucleotides, the binding

tendency of monomeric CAR is greatly reduced. All these binding motifs have been identified in the phenobarbital response element (PBREM) commonly found in CAR target genes [80]. Once bound, activated CAR will recruit coactivators/corepressors to modulate gene expression (Table 8.3).

Although this chapter has concentrated on the role(s) of CAR in xenobiotic metabolism, it is important to remember that CAR also has many endogenous functions. It is particularly important in control of lipid metabolism and energy homeostasis, appearing to act as an energy sensor, and influencing metabolism accordingly [17,81–86]. CAR is also involved in the control of bilirubin metabolism and heme biosynthesis, as well as bile acid and steroid/thyroid hormone homeostasis. For a recent review of this area, see di Masi *et al*. [15]. The range of endogenous roles with which CAR is involved means it is vital to consider the potential for functional cross talk between endogenous control and xenobiotic metabolism. For instance, the energy state of an organism can have significant effects on the uptake and pharmacokinetics of a drug because nutritional state can influence the induction of P450s by CAR [17]. Other considerations relating to CAR-induced xenobiotic metabolism include differential regulation due to species specificity, gender-specific induction, and the circadian control. Species specificity is discussed in more detail later in this chapter. Circadian control is a potentially important determinant of CAR expression and induction. CAR exhibits a circadian expression profile in mice because of its regulation by the PAR-domain basic leucine zipper transcription factors DBP (albumin D-box binding protein), TEF (thyrotroph embryonic factor), and HLF (hepatic leukemia factor), all of which are regulated by core components of the circadian machinery [87]. This results in a temporal induction of CAR target genes. Since downstream genes, such as P450s, are likely to display circadian expression profiles as a result, it is understandable that the clinical profile of certain drugs, such as cyclophosphamide and mitoxantrone, improves when dosing time in terms of circadian cycle is considered [87]. This feature should always be considered when investigating compounds that are likely CAR interactors, particularly in mouse models.

Overall, CAR regulation of P450 expression is complex, involving structural activation, regulation by signaling pathways, and interaction with various factors influenced downstream by CAR, including energy metabolism. It is, therefore, important when investigating compounds that appear to induce P450s via a CAR-dependent mechanism to consider that the endogenous control of CAR and the way in which it influences other homeostatic mechanisms, such as energy homeostasis, can have a significant effect on P450 expression profile and thus xenobiotic metabolism.

8.2.3 Aryl Hydrocarbon Receptor

The AHR has been known significantly longer than PXR and CAR, having been first identified in 1976 as the cytosolic receptor mediating 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-dependent induction of aryl hydrocarbon hydroxylase activity [88]. However, unlike PXR and CAR, the AHR is not a nuclear receptor but is the only known ligand-activated member of the basic helix-loop-helix (bHLH)/*p*eriod (PER)-*a*ryl hydrocarbon receptor nuclear translocator (ARNT)-*s*ingle minded (Sim) (bHLH-PAS) family of transcription factors. AHR ligands can be classified as synthetic, for example, dioxins and halogenated polycyclic aromatic hydrocarbons, and naturally occurring, such as flavonoids, indoles, and arachidonic acid metabolites [6,22,89]. As

a family, these proteins are implicated in the regulation of physiological processes, including organ development, metabolism, and stress and immune response [90]. However, one of the key roles of AHR is the control of xenobiotic metabolism.

AHR is responsible for the transactivational control of a variety of P450s (Table 8.1), most notably CYP1A1, CYP1A2, and CYP1B1, with CYP1A1 being considered a model enzyme for studying AHR-mediated gene activation. In order to modulate transcription, AHR heterodimerizes with its binding partner ARNT [33]. The transcriptional activation domains (TADs) of both molecules, once activated by ligands, activate transcription by inducing DNA binding via a subdomain N-terminal to the HLH domain. Both AHR and ARNT possess one TAD in the C-terminal region, with both containing glutamine and hydrophobic residues. However, the TAD of AHR also has acidic characteristics, whereas that of ARNT is rich in serine, threonine, and proline [91]. It is worth noting that while the N-terminal domain of AHR is well conserved across species, the C-terminal domain, and thus the TAD, is significantly divergent (58% identity between human and mouse forms) [92–94]. As a consequence, there are significant species differences in response to ligands (see Section 8.3.2 for details). Following activation, the AHR/ARNT heterodimer binds to the xenobiotic response element (XRE) in the promoter and/or enhancer regions of target genes (Fig. 8.1). Unlike classical bHLH proteins, the AHR/ARNT heterodimer binds to an asymmetric recognition site, binding to the core sequence of the XRE—*TNG*C*GTG* [92,95,96]. It has also been suggested that nucleotides adjacent to, but not part of, the core binding sequence are important for regulation of some genes [95]. Following DNA binding, AHR/ARNT recruits coactivators (Table 8.3) and other proteins, such as Mediator D and TFIID, resulting in chromatin remodeling and recruitment of RNA polymerases and in the initiation of gene expression (for a review of DNA binding, see Ref. 95).

Nuclear translocation is also a key step in the control of AHR-induced transcriptional regulation. AHR protein, which is inherently unstable in the absence of ligand, is stabilized in the cytoplasm by a complex consisting of two molecules of HSP90 and one each of hepatitis B virus X-associated protein 2 (XAP2) and the co-chaperone p23 [97–99]. The E3 ubiquitin ligases, C-terminal of HSP70 interacting protein (CHIP), and Cullin 4B also bind to AHR, which could explain the rapid degradation of free, monomeric AHR within cells [97]. In response to a molecular trigger, AHR dissociates from the core complex and localizes to the nucleus where it heterodimerizes with its binding partner to activate gene expression. At present, the signals regulating nuclear translocation are still being elucidated; however, there appears to be at least two separate mechanisms depending on whether the receptor is activated by ligand or an endogenous signal.

HSP90 has an important role in maintaining AHR stability and structure, with the central region binding to both the bHLH and PAS domains of AHR. HSP90 appears to be essential for maintaining AHR in its ligand-binding conformation, with AHR losing its dioxin-induced activity when HSP90 is disrupted *in vitro* [100–102]. Ligand binding appears to disrupt the binding of HSP90 to the PAS domain, which is adjacent to or just within the LBD, but not to the bHLH region, resulting in a conformational change that retains HSP90 binding during nuclear translocation. To explain this phenomenon, McGuire *et al*. [101] postulate that after ligand binding, HSP90 promotes a conformational change to accommodate the binding by AHR's dimerization partner, ARNT, following translocation to the nucleus, and therefore remains bound to AHR through the bHLH domain during nuclear shuttling. XAP2 is a 38-kDa protein with

significant homology to the immunophilins, such as FKBP52 [99]. Although not absolutely required for the structural integrity of the cytoplasmic core complex, XAP2 does appear to have a significant role in maintaining cytoplasmic localization [103]. In mice, nuclear localization appears to rely on importin β, which recognizes the bipartite NLS found in AHR. In the cytoplasm, XAP2 binding in the core complex masks the NLS, preventing importin binding and subsequent nuclear localization [103]. Ligand binding induces a conformational change in XAP2, resulting in dissociation of XAP2 and HSP90 from AHR. This unmasks the bipartite NLS, making it available for importin binding and thus nuclear import [98,104]. However, XAP2 does not seem to fulfill the same role in humans, as it appears to shuttle into the nucleus still bound to AHR [105,106]. This mechanism also involves PKC-mediated phosphorylation of two serine residues adjacent to the NLS—Ser12 and Ser36—which inhibits nuclear translocation by masking the NLS from importins [107].

Although ligand-dependent nuclear translocation seems to rely on ligand-induced conformational change of XAP2 and HSP90, evidence for another pathway involving cyclic-nucleotide-dependent signaling has been reported [97,98,108,109]. cAMP has been shown to interact with the AHR core complex and induce nuclear translocation [109]. However, the conformational change induced by this interaction reduced the ability of AHR to dimerize with ARNT, changed the DNA recognition sequence of AHR, and reduced dioxin-induced target expression [98,109].

Following nuclear translocation, it has been reported that phosphorylation of AHR and ARNT is essential for heterodimerization and thus DNA-binding activity [22]. The kinases involved were PKC and members of the ERK/MEK signaling pathway. This once again illustrates the importance of cooperative control with endogenous signaling pathways in xenobiotic metabolism. A novel nuclear binding partner has also been identified, which acts through the cAMP-related signaling pathway: the RelB subunit of NF-κB [110–112]. This heterodimer behaves in a PKA phosphorylation-dependent manner, with AHR DNA binding identified in the promoter region of interleukin-8. Although this alternative binding partner has a role in coregulation of inflammatory genes, acting in concert with the NF-κB pathway, any possible role in P450 regulation has yet to be investigated.

In summary, AHR is a promiscuous receptor that has a key role in metabolic response to environmental and dietary toxins. The mechanisms regulating AHR-induced P450 regulation are complex, requiring cross talk between many signaling and physiological processes. It is worthy to note that the majority of AHR research to date has been conducted *in vitro*, especially using murine cell lines. This has a number of limitations, including, in particular, AHR function is linked with a number of physiological processes, therefore important interactions will be missed and there are significant species differences in the structure and function of AHR. The application of AHR-humanized mice could be of use to further establish the *in vivo* regulation of this signaling pathway [113].

8.2.4 Receptor Cross Talk

In order to maintain a robust response to chemical challenge, multiple transcription factors have evolved to modulate P450 expression. This has created a complex interacting network of gene regulation [114]. The cross talk between the transcription factors, both in the ligands with which they interact and the downstream genes that they regulate,

allows the systems to be fine-tuned for the detoxification of specific compounds and the interactions between endogenous and exogenous pathways to be controlled. It is, therefore, imperative to understand the degree to which this phenomenon controls both *in vitro* and *in vivo* drug-metabolizing enzymes. This section discusses the cross talk between the three main transcription factors and the regulation of certain key P450s involved in drug metabolism.

8.2.4.1 CYP1A1/2. The CYP1A enzymes are associated with the metabolism of environmental toxins and aromatic hydrocarbons, providing protection against acute dioxin-induced hepatocellular necrosis and hepatic inflammation [4,5,115,116]. They are primarily under the control of AHR, with *Cyp1a1* and *Cyp1a2* possessing conserved XRE clusters in their proximal promoters [116]. However, other response elements have been located in the promoters of these genes, suggesting regulatory roles for other transcription factors. Also, PXR ligands can induce AHR target genes through the transcriptional regulation of AHR [4]. Yoshinari *et al*. [5] recently provided evidence that binding of the hCAR/RXRα heterodimer to a conserved ER8 motif within the proximal promoter of the CYP1A1 and CYP1A2 genes increases their expression. This finding suggests that drugs that activate CAR could alter the metabolism of CYP1A1 and CYP1A2 substrates and therefore be a source of drug–drug interactions. Glucocorticoid response elements (GREs) have also been identified in the promoter region of the rat *CYP1A1* gene, which, once activated, interact with the initiation complex to enhance AHR-induced transcription [54]. However, regulation by this mechanism appears to be species specific and does not occur in humans.

Inflammation has been reported to negatively regulate CYP1A1 expression, with exposure to UV-B repressing the induction of CYP1A1 immediately following irradiation, although CYP1A1 is induced in an AHR-dependent manner several hours following irradiation [115,117]. This was attributed to direct interaction between AHR and the NFκB RelA (p65) subunit, interfering with cofactor recruitment to the promoter. The repression of Cyp1a1 expression in response to oxidative stress had been reported by Morel and Barouki, who identified nuclear factor 1 (NF1) as the mediator of this effect [118]. Reciprocal cross talk between AHR and Nrf2 has also been recently reported, with XRE sequences being located in the Nrf2 promoter [12], and ARE sequences in the AHR promoter [119]. It has been suggested that a functional protein interaction may also exist between these molecules, although no data is currently available [120]. The reciprocal cross talk between these two systems further highlights the key role of AHR in the protection of the body from damage caused by electrophilic metabolites.

Regulation of CYP1A expression is therefore controlled by a number of core interacting transcription factors. However, because of the many endogenous roles played by the AHR, they are also subject to regulatory control by diverse signaling pathways, including those involved with inflammation and oxidative stress response, playing a key role in protecting the body from xenobiotic challenge.

8.2.4.2 CYP2B6. CYP2B6 metabolizes a wide range of drugs, including cyclophosphamide, valproic acid, ketamine, aminopyrine, methadone, and bupropion [59]. Its overall role in drug metabolism in man, however, has been considered minor [59,121]. CYP2B6 is induced by activators of hCAR, with the activated hCAR/RXRα heterodimer binding to the DR4 motif in the phenobarbital-responsive-element module

(PBREM) in the promoter [54,59]. However, cross talk with human pregnane X receptor (hPXR) has also been implicated in CYP2B6 regulation, through binding to the DR4 motif, all be it with lower affinity. A response element termed the *distal xenobiotic responsive enhancer module* (XREM) has also been identified as activated by either hCAR/RXRα or hPXR/RXRα [122]. This element works synergistically with the activated PBREM [59,122].

Although cross talk with PXR is reported to be part of the mechanism in the control of CYP2B6, other receptors are also implicated. For instance, in rodents, the glucocorticoid receptor (GR) directly activates CYP2B/Cyp2b expression [123,124]. Although the GR does not directly activate CYP2B6 in humans, it can affect CYP2B6 expression indirectly through induction of CAR and PXR [59]. Recent studies from the Negishi group have suggested that for maximal activity, the CYP2B6 promoter can be synergistically activated by the PBREM and a novel 52-bp response element, known as the *okadaic acid response element* ($OARE_{KI}$) (Fig. 8.2) [125]. The $OARE_{KI}$ contains a DR1 motif, which possesses an HNF4α binding site, a CACCC motif, which binds early growth response 1 (EGR1) protein, and an E box motif. EGR1 binding is required for maximal CAR-induced CYP2B6 activity, facilitating CAR/HNF4α cross talk by inducing DNA looping to bring the proximal $OARE_{KI}$ into proximity with the distal PBREM [126]. A recent study has demonstrated that the CCAAT/enhancer binding protein α (C/EBPα) and HNF4α cooperate with CAR to control CYP2B6 expression [121]. HNF4α binding to a proximal element located in the $OARE_{KI}$ (−217 bp) favors

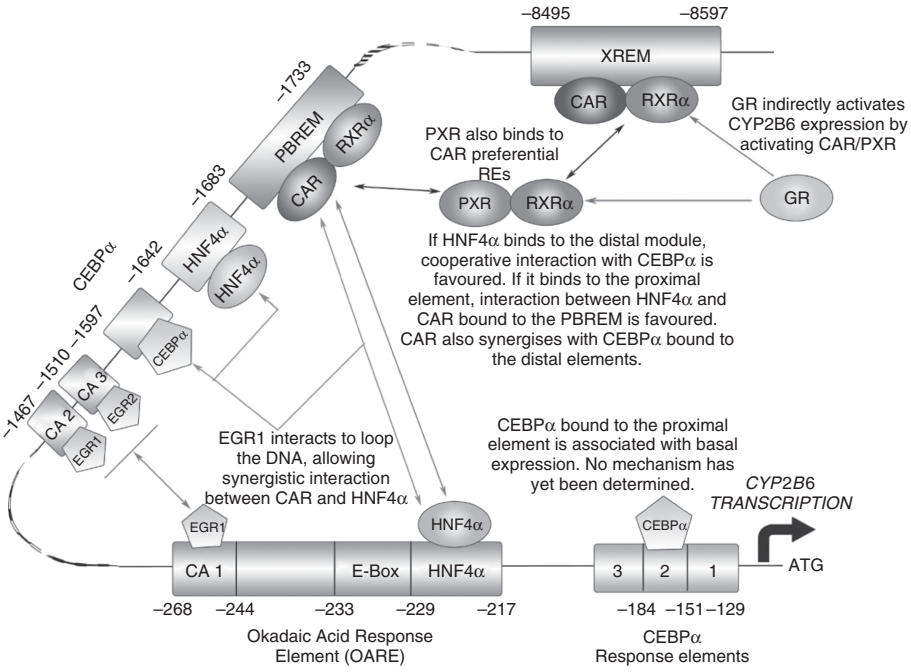


Figure 8.2 Regulatory network controlling CYP2B6 expression. *Source*: Adapted from Benet *et al*. [121], with additional information from Inoue and Negishi [126] and Wang and Tompkins [59]. (See color insert.)

CAR interaction, whereas binding to the distal region (−1642 bp) favors interaction with C/EBPα (Fig. 8.2). Variation in expression and binding of HNF4α and C/EBPα to the CYP2B6 promoter has been correlated to interindividual variations in CYP2B6 expression and activity, suggesting their importance in the regulation of this gene [121]. It is clear that CAR-induced transcriptional regulation of CYP2B6 involves intricate cross talk between CAR, HNF4α, EGR1, and C/EBPα as a minimum. However, the role of PXR in this mechanism remains to be clarified.

8.2.4.3 CYP2C8. *CYP2C8* is the most inducible gene in the CYP2C family, with a role in the metabolism of drugs such as rosiglitazone, paclitaxel, cerivastatin, and chloroquine, in addition to endogenous compounds, such as arachidonic acid [9]. The regulation of this protein remains poorly characterized, although DR4 binding at −8.8 kb is needed for CAR and PXR-mediated CYP2C8 induction [9]. Both CAR and PXR can bind to this element in response to ligands. However, Ferguson *et al*. [9] found that this response is not apparent when the reporter gene assay is performed in the HepG2 cell line but is seen when using metabolically competent primary human hepatocytes, demonstrating the limitations of HepG2 cells for drug induction studies. As with CYP2B6, basal expression of CYP2C8 involves HNF4α binding to a DR1 motif in the basal promoter region. A second functional HNF4α binding site has also been identified in the proximal promoter region [127]. Site-directed mutagenesis of the CYP2C8 promoter has shown that both HNF4α sites are important for RIF-induced PXR-mediated expression of CYP2C8, with that in the proximal promoter region being essential. Also, silencing of HNF4α using RNA interference (RNAi) decreased basal CYP2C8 reporter activity by 48% and abolished RIF-mediated induction. However, this treatment also repressed expression of CAR and PXR by 60% and 40%, respectively [127]. In light of the current limited information, further studies on the role of CAR in CYP2C8 control are required.

In addition to the above regulatory pathways, the GR also appears to be involved in the induction of members of the CYP2C gene family, with a functional GRE being located in the proximal promoter region in many CYP2C genes [9,54]. Ferguson *et al*. [9] demonstrated that CYP2C8 induction by the prototypical GR activator, dexamethasone, occurs by direct interaction with the GRE located in the promoter region of the gene, and not by interaction with PXR or CAR. A recent study in HepG2 cells identified retinoic-acid-receptor-related orphan receptor (ROR) response elements (ROREs) in the promoter of CYP2C8, which are activated by RORα1, RORα4, and RORγ1 isoforms [128]. The importance of RORs in the control of CYP2C8 was further supported by the finding that knockdown of the three isoforms resulted in a 50% decrease in *CYP2C8* mRNA. Since natural agonists of these receptors include cholesterol and its metabolites, CYP2C8 expression is likely to be influenced by normal physiological processes. These receptors are also linked to the control of circadian rhythm and therefore could be associated with circadian expression of the CYP2C genes. In summary, the regulatory control of CYP2C8 is still poorly understood, although certain key factors have been identified. More research is therefore required to integrate these signals into an understandable network and to more fully characterize regulation by CAR and PXR.

8.2.4.4 CYP2C9. CYP2C9 is the most highly expressed of the CYP2C family [129], with identified substrates including tolbutamide, diclofenac, and *S*-warfarin [130]. In

human hepatocytes, CYP2C9 could be regulated with prototypical PXR, CAR, and GR activators (RIF, PB, and dexamethasone, respectively), indicating that all three receptors have a role in the regulation of this enzyme [131]. Subsequent studies suggested that CAR plays a role in the basal expression of CYP2C9, with constitutive expression in HepG2 cells increasing with stable transfection of mouse or hCAR [131,132]. Two DR5 response elements, in a configuration similar to that of the PBREM and XREM modules, are located in the proximal promoter at −2899 bp and preferentially bind CAR over PXR [132]. A DR4 CAR/PXR binding motif at −1839 bp has also been identified [131]. Mutagenesis of these binding sites provided evidence that both are sensitive to CAR activation [132]. The induction of CYP2C9 by treatment with RIF, hyperforin, and PB has been shown to be mediated by hPXR binding to the DR4 response element [10,133]. The proximal DR4 binding site is therefore essential for activation, with the distal site acting in a cooperative capacity.

HNF4α plays an essential central role in the regulation of CYP2C9, with HNF4α binding sites located at −211, −185, and −152 bp. The site at −185 bp plays a central role in HNF4α-induced CYP2C9 expression and CAR/PXR synergism [127,134]. The HNF4α binding sites cooperate with the CAR/PXR responsive DR4 motif for maximal gene expression [127]. The underlying mechanism appears to be as a result of recruitment of a number of coactivators to the promoter, including cAMP response element binding protein (CBP), PGC-1α, PRIP-interacting protein with interacting methyltransferase (PIMT) domain, and nuclear receptor coactivator 6 (NCOA6) [135]. Current evidence suggests that NCOA6 forms a bridge between CAR and HNF4α bound to their respective response elements by direct interaction with the LXXLL motifs of each. NCOA6 therefore acts as a mediator promoting synergistic cross talk between CAR and HNF4α. It is not yet clear whether the same mechanism applies to hPXR-mediated control.

Other pathways that do not appear to directly interact with CAR/PXR-induced CYP2C9 expression have also been detailed. An imperfect GRE sequence at −1675 bp allows direct activation of the promoter by the GR, which appears to act synergistically with CAR/PXR [132]. HNF3γ, also known as FOXA3, has been implicated in the regulation of CYP2C genes, being found to bind to HNF3γ response elements in the promoter region, although a mechanism has yet to be elucidated [136]. A study by Drocourt *et al*. [137] suggested that the VDR can also regulate CYP2C9 expression. However, they point out that the concentrations of 1α,25-dihydroxyvitamin D_3 required for this interaction are above the levels seen physiologically, and therefore, the relevance of this interaction is questionable. A recent study has also shown the transcription factors GATA-2, -4, and -6 induce CYP2C9 through direct interaction with response elements in the promoter [138]. Again, any potential cross talk with other motifs has yet to be investigated, but the tissue-specific nature of GATA transcription factors could make this an interesting pathway when considering gene expression in extrahepatic tissues.

The regulation of CYP2C9 appears unusual in comparison to the other commonly inducible P450s. Although, HNF4α is again central to promoter activation, regulation by CAR and PXR is such that constitutive expression appears to be mediated through a CAR-mediated pathway, whereas ligand induction occurs through a PXR-mediated pathway, showing a more physiologically cooperative cross talk between these receptors than is often seen. Cross talk mediated through cofactors is also apparent in the mechanism underlying cooperation of CAR and HNF4α through mediation by NCOA6.

8.2.4.5 CYP3A4. CYP3A4 is the major P450 expressed in human liver and is responsible for the metabolism of approximately 60% of current drugs. The variation in CYP3A4 expression due to enzyme induction is therefore a major determinant of drug–drug interactions [39]. The key regulator of CYP3A4 expression is PXR, with the hPXR/RXRα heterodimer binding to an ER6 PXRE motif located at −172 bp relative to the transcription start site in the proximal promoter region [139–141]. Mutation of this element led to a 50% reduction in RIF-mediated response, indicating that this motif acts cooperatively with other regulatory elements [140]. A distal XREM enhancer has been identified at approximately −7800 bp, consisting of three nuclear-receptor-binding sites, dNR1-3. Disruption of the PXRE in the proximal promoter and the dNR1 imperfect DR3 binding motif in the distal XREM represses xenobiotic response by approximately 85%, indicating that both elements are essential for CYP3A4 expression. The dNR3 site is also required for maximal gene expression [140]. A further polymorphic enhancer module, known as the *constitutive liver enhancer module of CYP3A4* (CLEM4), has been identified at −11.4 kbp [142]. This is also required for maximal gene expression. This module contains multiple cis-acting elements, including binding sites for HNF-1, USF-1, AP-1, and HNF4α, as well as a perfect ER6 PXR binding motif. This enhancer region acts synergistically with the distal XREM and proximal promoter in PXR-mediated *CYP3A4* induction [143]. This region also appears to be involved in constitutive CYP3A4 expression [142]. However, the motif located in this region has been found to be significantly less competitive for PXR/RXRα binding relative to motifs found in the distal XREM and proximal promoter, instead the motif has a higher affinity for a PXR homodimer *in vitro* [143]. This homodimer has yet to be described *in vivo*, but potentially, this could result in a previously uncharted regulatory mechanism associated with CYP3A4 control.

All PXR binding motifs described above have also been shown to bind hCAR, although binding to the proximal promoter region alone is insufficient for CAR-induced expression. Therefore, evidence suggests that CYP3A4 is coordinately regulated by hCAR and hPXR [144,145]. Although PXR/CAR cooperation appears to be the main regulatory pathway connected to ligand-induced control of CYP3A4, neither are essential for constitutive expression, with individual disruption of these genes *in vivo* having no influence on basal CYP3A4 expression [142]. Istrate *et al*. [146] have used gel shift and LS174T-based reporter gene assays to show that a number of transcription factors can bind the ER6 and DR3 motifs, thus decreasing PXR-mediated gene induction through competition. For instance, the thyroid receptor (TR) α1 can bind to the response elements to repress CYP3A4 basal and ligand-induced expression. The ratio of PXR:TRα1 was moreover found to be correlated to basal expression level, suggesting a role in endogenous control. The VDR has also been found to compete with PXR for ER6, DR3, and DR4 binding sites to induce CYP3A4 expression in rat and human liver and intestinal slices, all with lower activity than PXR [137,147]. However, a recent study has shown that VDR and PXR can act synergistically in intestinal cell lines, apparently through VDR binding to the PXRE located in CLEM4 [148].

HNF4α also has a significant role in controlling CYP3A4 expression. Tirona *et al*. have identified a DR1, HNF4α binding site immediately upstream of the dNR1 and dNR2 binding sites in the XREM (−7783 bp). This was shown to be essential for ligand-induced promoter activity. *In vitro* reporter transactivation assays in HepG2, Caco-2 and HeLa cell lines indicated that basal and induced promoter activities are mediated through PXR/CAR, with the activity increasing synergistically in the presence of

HNF4α [149]. In contrast, the PXRE located in CLEM4 is flanked by two downstream HNF4α binding motifs [143], which suppress rather than facilitate PXR/CAR-induced gene expression as a consequence of HNF4α interference with PXR DNA binding. Liu *et al*. also reported that while disruption of the ER6 motif in the CLEM4 domain has no effect on basal expression, disruption of the DR3 motif in the XREM results in a decrease in basal activity. Other regulators of CYP3A4 expression have also been suggested. These include STAT1, HNF1, HNF3β (FOXA2), HNF3γ (FOXA3), and C/EBPα [150]. A systematic *in vitro* analysis in HepG2 and HuH7 cell lines has been performed by Bombail *et al*., in which putative binding sites were disrupted to identify functional interactions [151]. This study identified C/EBPα as a regulator of CYP3A4 expression, with mutation of the C/EBPα binding site at −132 bp causing a 60% decrease in basal expression and a decrease in I_{max} associated with RIF-, PB-, and metyrapone-mediated activation. In a previous work, two distal C/EBPα sites (located at −1402 and −1668 bp) were identified in the activation of CYP3A4 expression. HNF3γ is also a putative regulator, with a binding site located at −1730 bp. However, this receptor does not activate CYP3A4, but rather synergizes with C/EBPα, promoting gene expression by chromatin remodeling [151,152]. In addition to these findings, Bombail *et al*. reported that disruption of a putative Sp1 site at −104 to −97 bp resulted in a 50% decrease in promoter activity following PB treatment. Despite the presence of a putative GRE in the proximal promoter region, it is likely that GR agonists influence CYP3A4 expression by inducing PXR expression rather than interacting directly with the promoter [147,153]. CYP3A4 expression is also influenced by various physiological processes, as demonstrated by its regulation by FOXA2 and FOXA3, which have numerous endogenous roles, including embryonic development, organogenesis, and glucose homeostasis [154]. Other endogenous modulators include the HIF-1α transcription factor, which is upregulated in response to oxidative stress, and interacts to downregulate drug-metabolizing enzymes, including CYP3A4, in order to prevent the production of reactive oxygen species [155]. This seems to act through an indirect mechanism, such as downregulation of PXR and CAR, rather than through direct promoter binding. It is also important to remember that these transcription factors are subject to control by endogenous signaling molecules. This is highlighted by a recent report linking CYP3A4 expression to the cell cycle via phosphorylation of PXR by cyclin-dependent kinase 2 [156].

As this section has shown, although each of the enzymes discussed is preferentially controlled by a given transcription factor or nuclear receptor, a large variety of signaling pathways and coregulating molecules interact to control gene expression. The redundancy inherent in this system could be explained by the need to adapt to a wide range of environmental demands and endogenous stimuli. Our understanding of the interactions underlying transcriptional control is rapidly increasing. However, the networks we have currently identified are simplistic, omitting key endogenous signaling information that can have dramatic consequences for drug metabolism. While this section has aimed to give a broad overview of interaction at the level of P450 promoter regulation, it has of necessity excluded another level of functional cross talk, which exists between different nuclear receptors and transcription factors. Several reviews are now available, which discuss functional cross talk between nuclear receptors and certain transcription factors [15,16,114]. This interaction can significantly affect downstream regulation of target genes and thus the overall P450 expression profile.

8.3 OTHER CONSIDERATIONS FOR TRANSCRIPTIONAL REGULATION OF CYTOCHROME P450s

8.3.1 Methodological Challenges

Understanding P450 regulation is a vital step toward understanding drug interactions. Although the mechanisms underlying P450 transcriptional regulation are becoming clearer, methodological difficulties have introduced other issues that must be considered when analyzing current evidence.

Although *in vitro* methods, such as reporter gene assays, have been used to identify P450-inducing agents and mechanisms of transcriptional control, cell-based systems are unable to fully model transcriptional activation. Traditional overexpression systems have limitations because PXR and CAR will spontaneously translocate to the nucleus, probably because the members of the cytoplasmic retention complex become saturated [28,157]. This removes one of the key mechanisms underlying transcriptional regulation. A further problem is that with the exception of primary hepatocytes and a handful of immortalized cell lines, such as the HepaRG model [158–160], the majority of cell lines do not express physiologically relevant levels of both the P450-related transcription factors or P450s themselves. Cells also have the disadvantage that they cannot model systemic interactions, a significant problem when transcriptional regulators are influenced by systemic cues, such as hormonal signaling and energy state.

The use of conventional animal models to study ligand induction of P450s is therefore common, being able to model induction in a more physiologically relevant manner. However, because of the divergence of the metabolic system between species, models do not accurately predict P450 induction in humans [161]. The consequences of this can be profound. For instance, from a pharmaceutical perspective, failing to consider potential differences in drug metabolism could result in drugs reaching clinical trials that are therapeutically ineffective or, much more importantly, toxic to humans [162]. Species specificity is therefore a major issue to be addressed when considering results from animal models.

8.3.2 Species Specificity

The mechanisms underlying species specificity in drug induction are complex. However, species differences in P450 induction can often be ascribed to differences in the amino acid sequence of the LBD of the transcription factor involved [7,163–168]. Changes in the activity of NLS sequences have also been associated with alterations in nuclear localization, resulting in aberrant gene transcription [92,105,106,169,170]. Lichti-Kaiser *et al*. [37] have also identified species specificity in the response of hPXR and mPXR (mouse pregnane X receptor) to the cAMP nucleotide signaling pathway. CYP3A4 expression was found to be synergistically increased on treatment with RIF and the PKA inhibitor H89, whereas it decreased following treatment with H89 and pregnenolone-16α-carbonitrile (PCN). However, the study compared data from HepG2 cells transduced with an adenoviral expression vector containing hPXR with that from primary mouse hepatocytes, which may influence the findings. In the case of AHR, species-specific differences in receptor protein stability (hAHR< mAHR) and histone deacetylase complex recruitment have been implicated in differential response [170]. Improved understanding of species differences in nucleocytoplasmic shuttling,

TABLE 8.4 Examples of Species-Specific Ligands for PXR and CAR

Drug	Interacts with	Species			
		Mouse EC_{50} (nM)	Action	Human EC_{50} (nM)	Action
PCN	PXR	200–700	+	>10,000	N/A
Rifampicin		Weak		200–3000	+
SR12813		4100	+	120–200	+
Hyperforin		NR		23	+
5β-Cholestan-3α,7α, 12α-triol		2500	+	5000	+
TCPOBOP	CAR	20–100	+	Weak	N/A
CITCO		Weak		25–304	+
5α-Androstan-3α-ol		250–1500	–	1000–>10,000	–
Meclizine		25	+	500–1000	–
5β-Pregnane-3,20-dione		670–3000	+	>10,000 ≫10,000	Weak+ –
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	AHR	0.6	+	0.4–9	+

Includes EC_{50} (nM) where data is available.
NR, not reported; +, agonist; –, antagonist (inverse agonist, CAR only).
PXR and CAR data adapted from Refs 15 and 16. AHR data adapted from [177] (mouse), [178] (human), and [179].

cell signaling pathways and subsequent activation mechanisms is therefore of central importance. The availability of mouse models humanized for both the receptors which mediate drug induction as well as the P450's involved can help circumvent many of these problems.

Although the precise mechanisms have yet to be fully elucidated, numerous P450 inducers have been identified which interact with transcription factors in a species-dependent manner. A selection of these ligands has been detailed in Table 8.4. These include numerous drugs, endogenous compounds, environmental contaminants and certain herbal medicines. Most of this data has been generated using *in vitro* reporter gene assays, which are a useful model for identifying species-dependent ligand effects on transactivation activity of transcription factors. However, there are certain ligands that have proved of particular importance in the study of receptor activation. The macrolide antibiotic RIF and PCN have been shown to be species-specific activators of PXR, with RIF activating hPXR and PCN activating mPXR [15,171–173]. In the same way, 6-(4-chlorophenyl)imidazo-[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) are species-specific activators of CAR, with CITCO preferentially activating hCAR and TCPOBOP activating mCAR [15,171,174]. All four compounds are high affinity ligands for their preferred receptor, showing significantly lower or no affinity for the other species receptor. They are therefore commonly used as prototypical inducers of CYP3A4 (hPXR), Cyp3a11 (mPXR), CYP2B6 (hCAR), and cyp2b10 (mCAR), respectively, to validate models of P450 transactivation. With regard to AHR, a study by Westerink *et al*. identified differential CYP1A inducers between rat and human cell lines [175], while Boutros *et al*. also suggested that TCDD, although not affecting core AHR genes such as the CYP1 family, does exert species-specific effects, having a binding affinity for hAHR 10-fold less than that of mAHR, which could significantly

affect downstream regulation of target genes [176]. In view of the marked differences that can exist between ligand-induced regulation of drug metabolism, understanding how emerging drugs interact with human receptors before clinical testing is vital.

In order to address this issue, a number of new animal models have been created, which are humanized for the metabolic enzymes and transcription factors involved in drug metabolism [174,180,181]. Several approaches have been used in the construction of such models. Katoh *et al*. [181] created chimeric mice in which the innate hepatocytes of an immunocompromised mouse (uPA$^{+/+}$/SCID) were replaced with >90% human hepatocytes *in vivo*, resulting in an artificially humanized liver. However, the drawback of this model is that because these models must be produced as required, it requires a ready supply of human hepatocytes, which are both costly and hard to source as well as having the innate interindividual variability seen in the human population. The technique may also be restricted under legislation regulating the use of animals in experiments in some countries. A more common approach is to introduce the human gene into the mouse genome, either under the control of its own promoter by random integration or by replacing the endogenous mouse gene [174,180,182]. This has the advantage that the mouse lines can be continuously bred as well as potentially crossed to other mouse lines carrying linked genes of interest. This potentially expands the number of criteria that can be studied in a single model and helps overcomei potential ethical and sourcing issues encountered with the chimeric models.

Many humanized models are now available, including a range of humanized P450 models, such as CYP2D6 and CYP3A4, to enable drug metabolism to be characterized. However, the key models from the perspective of P450 transcriptional regulation are those in which the transcription factors are humanized. Some of these models are reviewed by Stanley *et al*. [16] and Gonzalez and Shah [180]. A battery of PXR and CAR models have now been developed to allow PXR- and CAR-mediated P450 induction to be dissected [174,183,184]. These include a model in which both PXR and CAR have been replaced with the human genes under the control of the corresponding endogenous promoters, as well as models in which one receptor is humanized while the other is deleted. Together with more traditional single humanized and knockout models, these models have the potential to allow species specificity and the role of receptor cross talk in P450 induction to be fully analyzed. A further advantage of these models is that their design allows expression of PXR and CAR splice variants at physiologically relevant levels, allowing interindividual variability to be studied [183] (Section 8.8.3). These models therefore provide a powerful tool for use in the analysis of mechanisms underlying species-specific regulation of P450s. Although not perfect, with receptors still under the influence of the mouse physiological environment, the data that can be gained from these humanized models is more relevant to humans than traditional animal models, especially since the majority of species-specific activity is thought to depend on the sequence of the LBD. As these models become more complex, with multiple genes being humanized in one model, data quality is likely to improve.

8.3.3 Splice Variants

Alternative splicing of mRNA is ubiquitous in eukaryotes, and many human genes are subject to the process, with 86% having a minor isoform frequency of 15% or greater, resulting in the production of different mRNAs, thus potentially generating multiple proteins from a single gene and greatly increasing genomic diversity [185]. There are

a number of different mechanisms involved in alternative splicing of mRNA, including exon skipping, intron retention, and the use of alternative splice donor and acceptor sites [186,187].

Alternative splicing of human P450 mRNAs was first described in the 1980s [188]. However, the identification of splice variants of hCAR and hPXR was more recent. Different-sized hPXR mRNAs were first identified in the late 1990s [189,190], with Fukuen *et al*. describing a total of 9 hPXR SVs whose expression levels varied significantly across a panel of 15 liver samples [191]. Further work to define the expression of hPXR SVs found the most common variants to be PXR.2 (lacking 111 nt) and PXR.3 (lacking 123 nt), representing approximately 6.7% and 0.32%, respectively, of hPXR mRNA in human liver and also found in a number of other tissues, including brain, heart, colon, and bone marrow [3]. PXR.2 was subsequently reported not to function in a transactivation assay because of the failure of ligands to bind to the LBD in a productive manner, probably as a result of a change in protein structure and the continued binding of corepressors [192].

Choi *et al*. in describing murine CAR, also reported the existence of a variant mRNA (mCAR2), which lacked exon 8 and was unable to function in a transactivation assay [45]. The first SVs of the hCAR gene were reported in 2003 when Savkur *et al*. described an mRNA for hCAR with a 4-amino-acid insert between exons 6 and 7 and a 5-amino-acid insert between exons 7 and 8 resulting from the use of alternative splice acceptor sites, and another with a complete deletion of exon 7 and loss of 39 amino acids [193]. In addition to these variants, Auerbach *et al*. [194] reported further hCAR SVs with one or other of the insertions between exons 6/7 and 7/8 and confirmed that such variants had compromised function. The range of hCAR SVs was extended dramatically when Lamba *et al*. reported 22 unique mRNAs from a panel of human liver and other tissues [195]. Several of these SVs had premature termination codons and failed to produce protein, but many had significant deletions or insertions, yielding proteins significantly different in size from the wild-type, or reference, form and which had altered N- and/or C-terminal amino acids. The hCAR SVs were differentially expressed, with only the hCAR reference form found in the intestine, whereas spleen, heart, and prostate expressed only the SVs, and were nonfunctional in transactivation assays, although the authors speculated that such SVs might possess biological function(s) yet to be identified [195]. A biological function was demonstrated for the hCAR SV with a 5-amino-acid insertion between exons 7 and 8, termed CAR3 by Auerbach *et al*., who found activity in a transactivation assay with an optimized 3× DR4 reporter using CITCO, thus defining CAR3 as a ligand-activated receptor in contrast to the reference form (CAR1) [196]. The same group further demonstrated that CAR2, with a 4-amino-acid insertion between exons 6 and 7, displayed a regulatory response distinct from that of CAR1, speculating that the ligand-binding pocket may be modified by the additional residues found in CAR2 [197]. Subsequently, CAR2 was also shown to be a ligand-activated receptor present in approximately 30% of total CAR transcripts in human hepatocytes and uniquely responsive to the plasticizer di(2-ethylhexyl) phthalate (DEHP) [198]. These latter findings—that a nuclear receptor present at significant levels in human liver (but absent in other animal models) is highly responsive to a chemical agent commonly found in a wide range of everyday materials, including medical devices—have potentially significant implications for safety testing in general and predictive human toxicity in particular.

To address these concerns, a unique panel of transgenic mice humanized for CAR and/or PXR have been generated, which are nulled for murine CAR and PXR and express the reference forms and two major SVs of hCAR (CAR2 and CAR3) and hPXR (PXR2 and PXR3) under the control of the endogenous mouse promotor [174]. Expression levels of the CAR and PXR SVs in these mouse models were comparable to that found in a panel of human livers, with the exception of hPXR2 in which the expression was approximately fourfold higher in the transgenic mouse [183]. The expression profile of the PXR SVs in the hPXR mouse found that PXR1 was expressed in a range of tissues at varying levels—liver > small intestine > kidney > lung > gonads > brain—while PXR2 was expressed, as a % of total PXR mRNA, at approximately the same level in all tissues, and PXR3 at highest levels in the kidney, gonads, and brain (Bower, CCM, unpublished observations). These mouse models provide a useful tool to investigate the role of CAR and PXR in drug metabolism, disposition, and efficacy in humans.

8.3.4 Genetic Polymorphisms

Interindividual variation in human drug metabolism is a significant cause of variability in drug response [199]; a main contributor is genetic polymorphism [200]. P450s are a highly polymorphic group of enzymes, with more than 350 different functional alleles so far identified in the genes, accounting for 40–50% of P450-dependent metabolism in humans [201,202]. Most of the characterized polymorphisms are variations in the coding sequence, such as SNPs, deletions, or gene amplifications [203,204]. They can, however, also be in gene promoters or intronic regions, which interfere with transcription factor binding or cause alternative or aberrant mRNA splicing [202,204]. Many of these polymorphisms manifest themselves as enzymes possessing altered activity [201]. One example of this is seen in the *CYP2B6*6* polymorphic isoform, which possesses two SNPs, 516Gly > Thr and 785Ala > Gly [59]. These substitutions result in alternative gene splicing, yielding a product with a deletion of exons 4–6 and that displays a significant reduction in catalytic activity and protein expression relative to the reference *CYP2B6*1*. Presence of these polymorphic variants can therefore significantly affect downstream drug metabolism.

Another source of interindividual variability in P450 expression is the polymorphisms in their regulators. One such polymorphic regulator is PXR, with 373 SNPs having been identified, and many of these proving to be functional [205,206]. As an example of the effect of PXR polymorphism on downstream gene expression, a study by Wang *et al*. [205] has examined the effect of two haplotypes of PXR commonly found in the Han Chinese population, H1 (TCAGGGGCCACC) and H2 (CCGAAAAC-TAAT), on P450 expression, using CYP3A4 activity as a marker. They found that those with the haplotype pair H1/H1 had much higher inducible CYP3A4 metabolic activity than those subjects with the H1/H2 and H2/H2 pairings following treatment with St Johns' Wort, as indicated by the significant differences in AUC_{0-t} and $AUC_{0-\infty}$ for the CYP3A4 probe drug nifedipine and its metabolite, dehydronifedipine. This phenomenon has been further confirmed by identification of functional polymorphisms in the FOXA2 transcription factor, which affect *CYP3A4* transcription and are also linked to diseases, such as type II diabetes [207]. Polymorphisms in P450s and the transcription factors, which control their expression, can therefore play a significant role in interindividual variation in xenobiotic metabolism. Understanding the genetic differences in P450 regulation remains an important subject for future study.

8.4 CONCLUSION

The adaptive response system that has been evolved by organisms to protect the cell from the many forms of toxic insult plays a central role in the metabolism and disposition of drugs. The importance of this system is underlined by its robust nature and built-in functional redundancy that allows at least a partial response in the absence or failure of a component part. The species differences observed in the regulation of P450 expression underline the importance of developing relevant models to test for human drug safety and efficacy, and in which factors such as gender, circadian rhythm, diet may also be taken into account. It is thus clear that an understanding of how drug metabolism is regulated, and the multitude of variable factors involved, is crucial to the optimal application of drug treatments and minimization of unwanted side effects and drug–drug interactions.

ABBREVIATIONS

AF-2	Activation Function 2
AHR	Aryl Hydrocarbon Receptor
AP-1	Activator Protein 1
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
bHLH	Basic Helix-Loop-Helix
bp	Base Pair
cAMP	cyclic AMP
CAR	Constitutive Androstane Receptor
CCRP	Cytoplasmic CAR Retention Protein
Cdk2	Cyclin-Dependent Kinase 2
C/EBPα	CCAAT/Enhancer Binding Protein α
ChIP	Chromatin Immunoprecipitation
CHIP	C-terminal of HSP70 interacting protein
CITCO	6-(4-Chlorophenyl)imidazo[2,1-*b*]thiazole-5-carbaldehyde *O*-(3, 4-dichlorobenzyl)oxime
CLEM4	Constitutive Liver Enhancer Module of CYP3A4
CREB	cAMP Response Element Binding Protein
CYP	Cytochrome P450
DBP	Albumin D-Box Binding Protein
DEHP	Di(2-ethylhexyl) Phthalate
DR	Direct Repeat
EGR1	Early Growth Response 1
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Elements
HIF1α	Hypoxia Inducible Factor 1α
HLF	Hepatic Leukemia Factor
HNF1	Hepatic Nuclear Factor 1
HNF3β	Hepatic Nuclear Factor 3β
HNF3γ	Hepatic Nuclear Factor 3γ
HNF4α	Hepatic Nuclear Factor 4α
LBD	Ligand-Binding Domain

LBP	Ligand-Binding Pocket
NCOA6	Nuclear Receptor Coactivator 6
NCoR	Nuclear Receptor Corepressor
NF1	Nuclear Factor 1
NLS	Nuclear Localization Signal
nt	Nucleotide
$OARE_{KI}$	Okadaic Acid Response Element
P450	Cytochrome P450
PAS	PER-ARNT-Sim
PB	Phenobarbital
PBREM	Phenobarbital-Responsive Enhancer Module
PCN	Pregnenolone-16α-carbonitrile
PDE4A5	Phosphodiesterase 4A5
PGC1α	Peroxisome-Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
PIMT	PRIP-Interacting Protein with Interacting Methyltransferase Domain
PKA	Protein Kinase A
PP2A	Protein Phosphatase 2A
PRIP	Peroxisome-Proliferator-Activated Receptor (PPAR)-Interacting Protein
PXR	Pregnane X Receptor
PXRE	PXR-Response Element
RIF	Rifampicin
ROR	Retinoic-Acid-Receptor-Related Orphan Receptor
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RXR	Retinoid X Receptor
SMRT	Silencing Mediator of Retinoid and Thyroid Hormone Receptor
SNP	Single Nucleotide Polymorphism
Sp1	Specificity Protein 1
SRC	Steroid Receptor Coactivator
STAT1	Signal Transducers and Activators of Transcription 1
SV	Splice Variant
TAD	Transcription Activation Domain
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TCPOBOP	1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene
TEF	Thyrotroph Embryonic Factor
TR	Thyroid Receptor
USF-1	Upstream Stimulatory Factor 1
VDR	Vitamin D Receptor
XAP2	X-Associated Protein 2
XRE	Xenobiotic Response Element
XREM	Xenobiotic Responsive Enhancer Module

REFERENCES

1. Blumberg B, *et al*. SXR, a novel steroid and xenobiotic-sensing nuclear receptor. Genes Dev 1998;12(20):3195–3205.

2. Kliewer SA, *et al*. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell 1998;92(1):73–82.
3. Lamba V, *et al*. PXR (NR1I2): splice variants in human tissues, including brain, and identification of neurosteroids and nicotine as PXR activators. Toxicol Appl Pharmacol 2004;199(3):251–265.
4. Köhle C, Bock KW. Coordinate regulation of human drug-metabolizing enzymes, and conjugate transporters by the Ah receptor, pregnane X receptor and constitutive androstane receptor. Chem Biol Interact 2009;77(4):689–699.
5. Yoshinari K, *et al*. Constitutive androstane receptor transcriptionally activates human CYP1A1 and CYP1A2 genes through a common regulatory element in the 5'-flanking region. Chem Biol Interact 2010;79(2):261–269.
6. Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol 2003;43:309–334.
7. Tirona RG, *et al*. Identification of amino acids in rat pregnane X receptor that determine species-specific activation. Mol Pharmacol 2004;65(1):36–44.
8. Itoh M, *et al*. Induction of human CYP2A6 is mediated by the pregnane X receptor with peroxisome proliferator-activated receptor-gamma coactivator 1alpha. J Pharmacol Exp Ther 2006;319(2):693–702.
9. Ferguson SS, *et al*. Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4a. Mol Pharmacol 2005;68(3):747–757.
10. Sahi J, *et al*. Regulation of cytochrome P450 2 C9 expression in primary cultures of human hepatocytes. J Biochem Mol Toxicol 2009;23(1):43–58.
11. Rezen T, *et al*. Interplay between cholesterol and drug metabolism. Biochim Biophys Acta 2010;1814(1):146–160.
12. Miao J, *et al*. Functional inhibitory cross-talk between constitutive androstane receptor and hepatic nuclear factor-4 in hepatic lipid/glucose metabolism is mediated by competition for binding to the DR1 motif and to the common coactivators, GRIP-1 and PGC-1alpha. J Biol Chem 2006;281(21):14537–14546.
13. Beilke LD, *et al*. Constitutive androstane receptor-mediated changes in bile acid composition contributes to hepatoprotection from lithocholic acid-induced liver injury in mice. Drug Metab Dispos 2009;37(5):1035–1045.
14. Moreau A, *et al*. Constitutive androstane receptor-vitamin D receptor crosstalk: consequence on CYP24 gene expression. Biochem Biophys Res Commun 2007;360(1):76–82.
15. di Masi A, *et al*. Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects. Mol Aspects Med 2009;30(5):297–343.
16. Stanley L, *et al*. PXR and CAR: nuclear receptors which play a pivotal role in drug disposition and chemical toxicity. Drug Metab Rev 2006;38(3):515–597.
17. Hernandez JP, Mota LC, Baldwin WS. Activation of CAR and PXR by dietary, environmental and occupational chemicals alters drug metabolism, intermediary metabolism, and cell proliferation. Curr Pharm Pers Med 2009;7(2):81–105.
18. Duret C, *et al*. Ketoconazole and miconazole are antagonists of the human glucocorticoid receptor: consequences on the expression and function of the constitutive androstane receptor and the pregnane X receptor. Mol Pharmacol 2006;70(1):329–339.
19. Sinz M, *et al*. Evaluation of 170 xenobiotics as transactivators of human pregnane X receptor (hPXR) and correlation to known CYP3A4 drug interactions. Curr Drug Metab 2006;7(4):375–388.
20. Staudinger JL, Ding X, Lichti K. Pregnane X receptor and natural products: beyond drug-drug interactions. Expert Opin Drug Metab Toxicol 2006;2(6):847–857.
21. Tolson AH, *et al*. Methadone induces the expression of hepatic drug-metabolizing enzymes through the activation of pregnane X receptor and constitutive androstane receptor. Drug Metab Dispos 2009;37(9):1887–1894.

22. Mukai R, *et al*. Suppression mechanisms of flavonoids on aryl hydrocarbon receptor-mediated signal transduction. Arch Biochem Biophys 2010;501(1):134–141. DOI: 10.1016/j.abb.2010.05.002.
23. Chrencik JE, *et al*. Structural disorder in the complex of human pregnane X receptor and the macrolide antibiotic rifampicin. Mol Endocrinol 2005;19(5):1125–1134.
24. Orans J, Teotico DG, Redinbo MR. The nuclear xenobiotic receptor pregnane X receptor: recent insights and new challenges. Mol Endocrinol 2005;19(12):2891–2900.
25. Watkins RE, *et al*. Coactivator binding promotes the specific interaction between ligand and the pregnane X receptor. J Mol Biol 2003;331(4):815–828.
26. Watkins RE, *et al*. The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. Science 2001;292(5525):2329–2333.
27. Timsit YE, Negishi M. CAR and PXR: the xenobiotic-sensing receptors. Steroids 2007;72(3):231–246.
28. Zelko I, *et al*. The peptide near the C terminus regulates receptor CAR nuclear translocation induced by xenochemicals in mouse liver. Mol Cell Biol 2001;21(8):2838–2846.
29. Kawana K, *et al*. Molecular mechanism of nuclear translocation of an orphan nuclear receptor, SXR. Mol Pharmacol 2003;63(3):524–531.
30. Squires EJ, Sueyoshi T, Negishi M. Cytoplasmic localization of pregnane X receptor and ligand-dependent nuclear translocation in mouse liver. J Biol Chem 2004;279(47): 49307–49314.
31. Kliewer SA, Goodwin B, Willson TM. The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. Endocr Rev 2002;23(5):687–702.
32. Wang CY, *et al*. Structural model reveals key interactions in the assembly of the pregnane X receptor/corepressor complex. Mol Pharmacol 2006;69(5):1513–1517.
33. Hankinson O. Role of coactivators in transcriptional activation by the aryl hydrocarbon receptor. Arch Biochem Biophys 2005;433(2):379–386.
34. Fallone F, *et al*. Retinoids repress Ah receptor CYP1A1 induction pathway through the SMRT corepressor. Biochem Biophys Res Commun 2004;322(2):551–556.
35. Hariparsad N, *et al*. Identification of pregnane-X receptor target genes and coactivator and corepressor binding to promoter elements in human hepatocytes. Nucleic Acids Res 2009;37(4):1160–1173.
36. Oshima M, *et al*. Molecular mechanism of transcriptional repression of AhR repressor involving ANKRA2, HDAC4, and HDAC5. Biochem Biophys Res Commun 2007; 364(2):276–282.
37. Lichti-Kaiser K, Xu C, Staudinger JL. Cyclic AMP-dependent protein kinase signaling modulates pregnane x receptor activity in a species-specific manner. J Biol Chem 2009; 284(11):6639–6649.
38. Lichti-Kaiser K, *et al*. A systematic analysis of predicted phosphorylation sites within the human pregnane X receptor protein. J Pharmacol Exp Ther 2009;331(1):65–76.
39. Plant N. The human cytochrome P450 sub-family: transcriptional regulation, inter-individual variation and interaction networks. Biochim Biophys Acta 2007;1770(3): 478–488.
40. Harmsen S, *et al*. The role of nuclear receptors in pharmacokinetic drug-drug interactions in oncology. Cancer Treat Rev 2007;33(4):369–380.
41. Harmsen S, *et al*. Nuclear receptor mediated induction of cytochrome P450 3A4 by anti-cancer drugs: a key role for the pregnane X receptor. Cancer Chemother Pharmacol 2009;64(1):35–43.
42. Ma X, Idle JR, Gonzalez FJ. The pregnane X receptor: from bench to bedside. Expert Opin Drug Metab Toxicol 2008;4(7):895–908.
43. Moore JT, Kliewer SA. Use of the nuclear receptor PXR to predict drug interactions. Toxicology 2000;153(1–3):1–10.

44. Baes M, *et al*. A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. Mol Cell Biol 1994;14(3): 1544–1552.
45. Choi HS, *et al*. Differential transactivation by two isoforms of the orphan nuclear hormone receptor CAR. J Biol Chem 1997;272(38):23565–23571.
46. Masahiko N, Honkakoski P. Induction of drug metabolism by nuclear receptor CAR: molecular mechanisms and implications for drug research. Eur J Pharm Sci 2000;11(4):259–264.
47. Maglich JM, *et al*. Nuclear pregnane X receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. Mol Pharmacol 2002;62(3):638–646.
48. Yamamoto Y, Kawamoto T, Negishi M. The role of the nuclear receptor CAR as a coordinate regulator of hepatic gene expression in defense against chemical toxicity. Arch Biochem Biophys 2003;409(1):207–211.
49. Dickins M. Induction of cytochromes P450. Curr Top Med Chem 2004;4(16):1745–1766.
50. Nakata K, *et al*. Nuclear receptor-mediated transcriptional regulation in phase I, II, and III xenobiotic metabolizing systems. Drug Metab Pharmacokinet 2006;21(6):437–457.
51. Tien ES, Negishi M. Nuclear receptors CAR and PXR in the regulation of hepatic metabolism. Xenobiotica 2006;36(10–11):1152–1163.
52. Petrick JS, Klaassen CD. Importance of hepatic induction of constitutive androstane receptor and other transcription factors that regulate xenobiotic metabolism and transport. Drug Metab Dispos 2007;35(10):1806–1815.
53. Tompkins LM, Wallace AD. Mechanisms of cytochrome P450 induction. J Biochem Mol Toxicol 2007;21(4):176–181.
54. Monostory K, Pascussi JM. Regulation of Drug-metabolizing human cytochrome P450s. Acta Chim Slov 2008;55(1):20–37.
55. Muntané J. Regulation of drug metabolism and transporters. Curr Drug Metab 2009;10(8):932–945.
56. Plant N, Aouabdi S. Nuclear receptors: the controlling force in drug metabolism of the liver? Xenobiotica 2009;39(8):597–605.
57. Moore DD. CAR: three new models for a problem child. Cell Metab 2005;1(1):6–8.
58. Wei P, *et al*. The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. Nature 2000;407(6806):920–923.
59. Wang H, Tompkins LM. CYP2B6: new insights into a historically overlooked cytochrome P450 isozyme. Curr Drug Metab 2008;9(7):598–610.
60. Dussault I, *et al*. A structural model of the constitutive androstane receptor defines novel interactions that mediate ligand-independent activity. Mol Cell Biol 2002;22(15): 5270–5280.
61. Kenakin T. Principles: receptor theory in pharmacology. Trends Pharmacol Sci 2004;25(4):186–192.
62. Moore DD, *et al*. International union of pharmacology. LXII. The NR1H and NR1I receptors: constitutive androstane receptor, pregnene X receptor, farnesoid X receptor a, farnesoid X receptor b, liver X receptor a, liver X receptor b, and vitamin D receptor. Pharmacol Rev 2006;58(4):742–759.
63. Hernandez JP, *et al*. Sexually dimorphic regulation and induction of P450s by the constitutive androstane receptor (CAR). Toxicology 2009;256(1–2):53–64.
64. Rencurel F, *et al*. AMP-activated protein kinase mediates phenobarbital induction of CYP2B gene expression in hepatocytes and a newly derived human hepatoma cell line. J Biol Chem 2005;280(6):4367–4373.
65. Suino K, *et al*. The nuclear xenobiotic receptor CAR: structural determinants of constitutive activation and heterodimerization. Mol Cell 2004;16(6):893–905.

66. Xu RX, *et al*. A structural basis for constitutive activity in the human CAR/RXRα heterodimer. Mol Cell 2004;16(6):919–928.
67. Kobayashi K, *et al*. Cytoplasmic accumulation of the nuclear receptor CAR by a tetratricopeptide repeat protein in HepG2 cells. Mol Pharmacol 2003;64(5):1069–1075.
68. Kanno Y, *et al*. Characterization of nuclear localization signals and cytoplasmic retention region in the nuclear receptor CAR. Biochim Biophys Acta 2005;1745(2):215–222.
69. Kanno Y, Miyazaki Y, Inouye Y. The nuclear import of the constitutive androstane receptor by importin/Ran-GTP systems. Biochim Biophys Acta 2010;1803(8):968–974.
70. Mutoh S, *et al*. Dephosphorylation of threonine 38 is required for nuclear translocation and activation of human xenobiotic receptor CAR (NR1I3). J Biol Chem 2009;284(50):34785–34792.
71. Kawamoto T, *et al*. Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. Mol Cell Biol 1999;19(9):6318–6322.
72. Yoshinari K, *et al*. Identification of the nuclear receptor CAR:HSP90 complex in mouse liver and recruitment of protein phosphatase 2A in response to phenobarbital. FEBS Lett 2003;548(1–3):17–20.
73. Koike C, Moore R, Negishi M. Extracellular signal-regulated kinase is an endogenous signal retaining the nuclear constitutive active/androstane receptor (CAR) in the cytoplasm of mouse primary hepatocytes. Mol Pharmacol 2007;71(5):1217–1221.
74. Blattler SM, *et al*. In the regulation of cytochrome P450 genes, phenobarbital targets LKB1 for necessary activation of AMP-activated protein kinase. Proc Natl Acad Sci U S A 2007;104(3):1045–1050.
75. Kanno Y, Inoue Y, Inouye Y. 5-aminoimidazole-4-carboxamide-1-beta-ribofuranoside (AICAR) prevents nuclear translocation of constitutive androstane receptor by AMP-activated protein kinase (AMPK) independent manner. J Toxicol Sci 2010;35(4):571–576.
76. Rencurel F, *et al*. Stimulation of AMP-activated protein kinase is essential for the induction of drug metabolizing enzymes by phenobarbital in human and mouse liver. Mol Pharmacol 2006;70(6):1925–1934.
77. Shindo S, Numazawa S, Yoshida T. A physiological role of AMP-activated protein kinase in phenobarbital-mediated constitutive androstane receptor activation and CYP2B induction. Biochem J 2007;401(3):735–741.
78. Koike C, Moore R, Negishi M. Localization of the nuclear receptor CAR at the cell membrane of mouse liver. FEBS Lett 2005;579(30):6733–6736.
79. Sueyoshi T, *et al*. PPP1R16A, the membrane subunit of protein phosphatase 1β, signals nuclear translocation of the nuclear receptor constitutive active/androstane receptor. Mol Pharmacol 2008;73(4):1113–1121.
80. Frank C, *et al*. Characterization of DNA complexes formed by the nuclear receptor constitutive androstane receptor. J Biol Chem 2003;278(44):43299–43310.
81. Ding X, *et al*. Regulation of constitutive androstane receptor and its target genes by fasting, cAMP, hepatocyte nuclear factor 4a, and the coactivator peroxisome proliferator-activated receptor g coactivator-1a. J Biol Chem 2006;281(36):26540–26551.
82. Finn RD, *et al*. Unsaturated fatty acid regulation of cytochrome P450 expression via a CAR-dependent pathway. Biochem J 2009;417(1):43–54.
83. Maglich JM, Lobe DC, Moore JT. The nuclear receptor CAR (NR1I3) regulates serum triglyceride levels under conditions of metabolic stress. J Lipid Res 2009;50(3):439–445.
84. Maglich JM, *et al*. The nuclear receptor CAR is a regulator of thyroid hormone metabolism during caloric restriction. J Biol Chem 2004;279(19):19832–19838.
85. Roth A, *et al*. Regulatory cross-talk between drug metabolism and lipid homeostasis: constitutive androstane receptor and pregnane X receptor increase Insig-1 expression. Mol Pharmacol 2008;73(4):1282–1289.
86. Wada T, Gao J, Xie W. PXR and CAR in energy metabolism. Trends Endocrinol Metab 2009;20(6):273–279.

87. Gachon F, *et al*. The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. Cell Metab 2006;4(1):25–36.
88. Poland A, Glover E, Kende AS. Stereospecific, high affinity binding of 2,3,7,8 tetrachlorodibenzo p dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. J Biol Chem 1976;251(16):4936–4946.
89. Denison MS, *et al*. Ligand binding and activation of the Ah receptor. Chem Biol Interact 2002;141(1–2):3–24.
90. Barouki R, Coumoul X, Fernandez-Salguero PM. The aryl hydrocarbon receptor, more than a xenobiotic-interacting protein. FEBS Lett 2007;581(19):3608–3615.
91. Schmidt JV, Bradfield CA. AH receptor signaling pathways. Annu Rev Cell Dev Biol 1996;12:55–89.
92. Ramadoss P, Perdew GH. The transactivation domain of the Ah receptor is a key determinant of cellular localization and ligand-independent nucleocytoplasmic shuttling properties. Biochemistry 2005;44(33):11148–11159.
93. Flaveny CA, Murray IA, Perdew GH. Differential gene regulation by the human and mouse aryl hydrocarbon receptor. Toxicol Sci 2010;114(2):217–225.
94. Flaveny CA, Perdew GH. Transgenic humanized AHR mouse reveals differences between human and mouse AHR ligand selectivity. Mol Cell Pharm 2009;1(3):119–123.
95. Swanson HI. DNA binding and protein interactions of the AHR/ARNT heterodimer that facilitate gene activation. Chem Biol Interact 2002;141(1–2):63–76.
96. Swanson HI, Tullis K, Denison MS. Binding of transformed Ah receptor complex to a dioxin responsive transcriptional enhancer: evidence for two distinct heteromeric DNA-binding forms. Biochemistry 1993;32(47):12841–12849.
97. de Oliveira SK, Smolenski A. Phosphodiesterases link the aryl hydrocarbon receptor complex to cyclic nucleotide signaling. Chem Biol Interact 2009;77(4):723–733.
98. Oesch-Bartlomowicz B, Oesch F. Role of cAMP in mediating AHR signaling. Chem Biol Interact 2009;77(4):627–641.
99. Petrulis JR, Perdew GH. The role of chaperone proteins in the aryl hydrocarbon receptor core complex. Chem Biol Interact 2002;141(1–2):25–40.
100. Coumailleau P, *et al*. Definition of a minimal domain of the dioxin receptor that is associated with Hsp90 and maintains wild type ligand binding affinity and specificity. J Biol Chem 1995;270(42):25291–25300.
101. McGuire J, *et al*. A cellular factor stimulates ligand-dependent release of hsp90 from the basic helix-loop-helix dioxin receptor. Mol Cell Biol 1994;14(4):2438–2446.
102. Pongratz I, Mason GGF, Poellinger L. Dual roles of the 90-kDa heat shock protein hsp90 in modulating functional activities of the dioxin receptor. Evidence that the dioxin receptor functionally belongs to a subclass of nuclear receptors which require hsp90 both for ligand binding activity and repression of intrinsic DNA binding activity. J Biol Chem 1992;267(19):13728–13734.
103. Petrulis JR, *et al*. The hsp90 Co-chaperone XAP2 alters importin beta recognition of the bipartite nuclear localization signal of the Ah receptor and represses transcriptional activity. J Biol Chem 2003;278(4):2677–2685.
104. Ikuta T, *et al*. Nuclear localization and export signals of the human aryl hydrocarbon receptor. J Biol Chem 1998;273(5):2895–2904.
105. Pollenz RS, Wilson SE, Dougherty EJ. Role of endogenous XAP2 protein on the localization and nucleocytoplasmic shuttling of the endogenous mouse Ah^{b-1}receptor in the presence and absence of ligand. Mol Pharmacol 2006;70(4): 1369–1379.
106. Ramadoss P, *et al*. Divergent roles of hepatitis B virus X-associated protein 2 (XAP2) in human versus mouse Ah receptor complexes. Biochemistry 2004;43(3):700–709.

107. Ikuta T, Kobayashi Y, Kawajiri K. Phosphorylation of nuclear localization signal inhibits the ligand-dependent nuclear import of aryl hydrocarbon receptor. Biochem Biophys Res Commun 2004;317(2):545–550.
108. de Oliveira SK, *et al*. Phosphodiesterase 2A forms a complex with the co-chaperone XAP2 and regulates nuclear translocation of the aryl hydrocarbon receptor. J Biol Chem 2007;282(18):13656–13663.
109. Oesch-Bartlomowicz B, *et al*. Aryl hydrocarbon receptor activation by cAMP vs. dioxin: divergent signaling pathways. Proc Natl Acad Sci U S A 2005;102(26):9218–9223.
110. Vogel CFA, Matsumura F. A new cross-talk between the aryl hydrocarbon receptor and RelB, a member of the NF- $\hat{I}^{\circ}$B family. Chem Biol Interact 2009;77(4):734–745.
111. Vogel CFA, *et al*. RelB, a new partner of aryl hydrocarbon receptor-mediated transcription. Mol Endocrinol 2007;21(12):2941–2955.
112. Vogel CFA, Sciullo E, Matsumura F. Involvement of RelB in aryl hydrocarbon receptor-mediated induction of chemokines. Biochem Biophys Res Commun 2007;363(3): 722–726.
113. Moriguchi T, *et al*. Distinct response to dioxin in an arylhydrocarbon receptor (AHR)-humanized mouse. Proc Natl Acad Sci U S A 2003;100(10):5652–5657.
114. Pascussi JM, *et al*. The tangle of nuclear receptors that controls xenobiotic metabolism and transport: crosstalk and consequences. Annu Rev Pharmacol Toxicol 2008;48:1–32.
115. Luecke S, *et al*. Cytochrome P450 1A1 gene regulation by UVB involves crosstalk between the aryl hydrocarbon receptor and nuclear factor kB. Chem Biol Interact 2010;184(3):466–473.
116. Nukaya M, Moran S, Bradfield CA. The role of the dioxin-responsive element cluster between the Cyp1a1 and Cyp1a2 loci in aryl hydrocarbon receptor biology. Proc Natl Acad Sci U S A 2009;106(12):4923–4928.
117. Fritsche E, *et al*. Lightening up the UV response by identification of the arylhydrocarbon receptor as a cytoplasmatic target for ultraviolet B radiation. Proc Natl Acad Sci U S A 2007;104(21):8851–8856.
118. Morel Y, Barouki R. Down-regulation of cytochrome P450 1A1 gene promoter by oxidative stress: critical contribution of nuclear factor 1. J Biol Chem 1998;273(41): 26969–26976.
119. Shin S, *et al*. NRF2 modulates aryl hydrocarbon receptor signaling: influence on adipogenesis. Mol Cell Biol 2007;27(20):7188–7197.
120. Hayes JD, Dinkova-Kostova AT, McMahon M. Cross-talk between transcription factors AhR and Nrf2: lessons for cancer chemoprevention from dioxin. Toxicol Sci 2009;111(2):199–201.
121. Benet M, *et al*. CCAAT/enhancer-binding protein a (C/EBPa) and hepatocyte nuclear factor 4a (HNF4a) synergistically cooperate with constitutive androstane receptor (CAR) to transactivate the human cytochrome P450 2B6 (CYP2B6) gene: application to the development of a metabolically competent human hepatic cell model. J Biol Chem 2010;285:28457–28471. DOI: 10.1074/jbc.M110.118364.
122. Wang H, *et al*. A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. J Biol Chem 2003;278(16):14146–14152.
123. Meehan RR, *et al*. Regulation of phenobarbital-inducible cytochrome P-450s in rat and mouse liver following dexamethasone administration and hypophysectomy. Biochem J 1988;254(3):789–797.
124. Schuetz EG, *et al*. The glucocorticoid receptor is essential for induction of cytochrome P-4502B by steroids but not for drug or steroid induction of CYP3A or P-450 reductase in mouse liver. Drug Metab Dispos 2000;28(3):268–278.
125. Inoue K, Negishi M. Nuclear receptor CAR requires early growth response 1 to activate the human cytochrome P450 2B6 gene. J Biol Chem 2008;283(16):10425–10432.

126. Inoue K, Negishi M. Early growth response 1 loops the CYP2B6 promoter for synergistic activation by the distal and proximal nuclear receptors CAR and HNF4a. FEBS Lett 2009;583(12):2126–2130.
127. Rana R, *et al*. Hepatocyte nuclear factor 4a regulates rifampicin-mediated induction of CYP2C genes in primary cultures of human hepatocytes. Drug Metab Dispos 2010;38(4):591–599.
128. Chen Y, *et al*. Identification of human CYP2C8 as a retinoid-related orphan nuclear receptor target gene. J Pharmacol Exp Ther 2009;329(1):192–201.
129. Miners JO, Birkett DJ. Cytochrome P4502C9: an enzyme of major importance in human drug metabolism. Br J Clin Pharmacol 1998;45(6):525–538.
130. Gerbal-Chaloin S, *et al*. Induction of CYP2C genes in human hepatocytes in primary culture. Drug Metab Dispos 2001;29(3):242–251.
131. Gerbal-Chaloin S, *et al*. Transcriptional regulation of CYP2C9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. J Biol Chem 2002;277(1):209–217.
132. Ferguson SS, *et al*. Regulation of human CYP2C9 by the constitutive androstane receptor: discovery of a new distal binding site. Mol Pharmacol 2002;62(3):737–746.
133. Chen Y, *et al*. Induction of human CYP2C9 by rifampicin, hyperforin, and phenobarbital is mediated by the pregnane X receptor. J Pharmacol Exp Ther 2004;308(2):495–501.
134. Chen Y, *et al*. The nuclear receptors constitutive androstane receptor and pregnane X receptor cross-talk with hepatic nuclear factor 4a to synergistically activate the human CYP2C9 promoter. J Pharmacol Exp Ther 2005;314(3):1125–1133.
135. Surapureddi S, *et al*. Nuclear receptor coactivator 6 mediates the synergistic activation of human cytochrome P-450 2 C9 by the constitutive androstane receptor and hepatic nuclear factor-4a. Mol Pharmacol 2008;74(3):913–923.
136. Bort R, *et al*. Role of hepatocyte nuclear factor 3 g in the expression of human CYP2C genes. Arch Biochem Biophys 2004;426(1):63–72.
137. Drocourt L, *et al*. Expression of CYP3A4, CYP2B6, and CYP2C9 is regulated by the vitamin D receptor pathway in primary human hepatocytes. J Biol Chem 2002;277(28):25125–25132.
138. Mwinyi J, *et al*. New insights into the regulation of CYP2C9 gene expression: the role of the transcription factor GATA-4. Drug Metab Dispos 2010;38(3):415–421.
139. Barwick JL, *et al*. Trans-species gene transfer for analysis of glucocorticoid-inducible transcriptional activation of transiently expressed human CYP3A4 and rabbit CYP3A6 in primary cultures of adult rat and rabbit hepatocytes. Mol Pharmacol 1996;50(1):10–16.
140. Goodwin B, Hodgson E, Liddle C. The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. Mol Pharmacol 1999;56(6):1329–1339.
141. Quattrochi LC, Guzelian PS. CYP3A regulation: From pharmacology to nuclear receptors. Drug Metab Dispos 2001;29(5):615–622.
142. Matsumura K, *et al*. Identification of a novel polymorphic enhancer of the human CYP3A4 gene. Mol Pharmacol 2004;65(2):326–334.
143. Liu FJ, *et al*. The far and distal enhancers in the CYP3A4 gene co-ordinate the proximal promoter in responding similarly to the pregnane X receptor but differentially to hepatocyte nuclear factor-4a. Biochem J 2008;409(1):243–250.
144. Goodwin B, *et al*. Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor. Mol Pharmacol 2002;62(2):359–365.
145. Urquhart BL, Tirona RG, Kim RB. Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. J Clin Pharmacol 2007;47(5):566–578.
146. Istrate MA, *et al*. Regulation of CYP3A4 by pregnane X receptor: the role of nuclear receptors competing for response element binding. Biochem Biophys Res Commun 2010;393(4):688–693.

147. Khan AA, *et al*. Comparison of effects of VDR versus PXR, FXR and GR ligands on the regulation of CYP3A isozymes in rat and human intestine and liver. Eur J Pharm Sci 2009;37(2):115–125.
148. Pavek P, *et al*. Intestinal cell-specific vitamin D receptor (VDR)-mediated transcriptional regulation of CYP3A4 gene. Chem Biol Interact 2010;79(2):277–287.
149. Tirona RG, *et al*. The orphan nuclear receptor HNF4a determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. Nat Med 2003;9(2):220–224.
150. Martínez-Jiménez CP, *et al*. Transcriptional regulation and expression of CYP3A4 in hepatocytes. Curr Drug Metab 2007;8(2):185–194.
151. Bombail V, *et al*. Role of Sp1, C/EBPa, HNF3, and PXR in the basal- and xenobiotic-mediated regulation of the CYP3A4 gene. Drug Metab Dispos 2004;32(5):525–535.
152. Rodríguez-Antona C, *et al*. Transcriptional regulation of human CYP3A4 basal expression by CCAAT enhancer-binding protein a and hepatocyte nuclear factor-3 g. Mol Pharmacol 2003;63(5):1180–1189.
153. Zimmermann C, *et al*. PXR-mediated induction of human CYP3A4 and mouse Cyp3a11 by the glucocorticoid budesonide. Eur J Pharm Sci 2009;36(4–5):565–571.
154. Friedman JR, Kaestner KH. The Foxa family of transcription factors in development and metabolism. Cell Mol Life Sci 2006;63(19–20):2317–2328.
155. Legendre C, *et al*. Drug-metabolising enzymes are down-regulated by hypoxia in differentiated human hepatoma HepaRG cells: HIF-1a involvement in CYP3A4 repression. Eur J Cancer 2009;45(16):2882–2892.
156. Lin W, *et al*. Cyclin-dependent kinase 2 negatively regulates human pregnane X receptor-mediated CYP3A4 gene expression in HepG2 liver carcinoma cells. J Biol Chem 2008;283(45):30650–30657.
157. Saradhi M, *et al*. Pregnane and Xenobiotic Receptor (PXR/SXR) resides predominantly in the nuclear compartment of the interphase cell and associates with the condensed chromosomes during mitosis. Biochim Biophys Acta 2005;1746(2):85–94.
158. Aninat C, *et al*. Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. Drug Metab Dispos 2006;34(1):75–83.
159. Guillouzo A, *et al*. The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. Chem Biol Interact 2007;168(1):66–73.
160. Hart SN, *et al*. A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. Drug Metab Dispos 2010;38(6):988–994.
161. Collins JM. Inter-species differences in drug properties. Chem Biol Interact 2001;134(3):237–242.
162. Powley MW, *et al*. Safety assessment of drug metabolites: implications of regulatory guidance and potential application of genetically engineered mouse models that express human P450s. Chem Res Toxicol 2009;22(2):257–262.
163. Graham MJ, Lake BG. Induction of drug metabolism: species differences and toxicological relevance. Toxicology 2008;254(3):184–191.
164. Jyrkkärinne J, *et al*. Amino acids important for ligand specificity of the human constitutive androstane receptor. J Biol Chem 2005;280(7):5960–5971.
165. LeCluyse EL. Pregnane X receptor: molecular basis for species differences in CYP3A induction by xenobiotics. Chem Biol Interact 2001;134(3):283–289.
166. Östberg T, *et al*. Identification of residues in the PXR ligand binding domain critical for species specific and constitutive activation. Eur J Biochem 2002;269(19):4896–4904.
167. Poso A, Honkakoski P. Ligand recognition by drug-activated nuclear receptors PXR and CAR: structural, site-directed mutagenesis and molecular modeling studies. Mini-Rev Med Chem 2006;6(8):937–943.

168. Repo S, *et al*. Ligand specificity of constitutive androstane receptor as probed by induced-fit docking and mutagenesis. J Med Chem 2008;51(22):7119–7131.
169. Kanno Y, *et al*. Difference in nucleocytoplasmic shuttling sequences of rat and human constitutive active/androstane receptor. Biochim Biophys Acta 2007;1773(6):934–944.
170. Suzuki T, Nohara K. Regulatory factors involved in species-specific modulation of aryl-hydrocarbon receptor (AhR)-dependent gene expression in humans and mice. J Biochem 2007;142(4):443–452.
171. Moore LB, *et al*. Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. J Biol Chem 2000;275(20):15122–15127.
172. Mu Y, *et al*. A pregnane X receptor agonist with unique species-dependent stereoselectivity and its implications in drug development. Mol Pharmacol 2005;68(2):403–413.
173. Mu Y, *et al*. Traditional Chinese medicines Wu Wei Zi (Schisandra chinensis Baill) and Gan Cao (Glycyrrhiza uralensis Fisch) activate pregnane X receptor and increase warfarin clearance in rats. J Pharmacol Exp Ther 2006;316(3):1369–1377.
174. Scheer N, *et al*. A novel panel of mouse models to evaluate the role of human pregnane X receptor and constitutive androstane receptor in drug response. J Clin Invest 2008;118(9):3228–3239.
175. Westerink WMA, Stevenson JCR, Schoonen WGEJ. Pharmacologic profiling of human and rat cytochrome P450 1A1 and 1A2 induction and competition. Arch Toxicol 2008;82(12):909–921.
176. Boutros PC, *et al*. Transcriptomic responses to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in liver: comparison of rat and mouse. BMC Genomics 2008;16(9):9.
177. Kronenberg S, Esser C, Carlberg C. An aryl hydrocarbon receptor conformation acts as the functional core of nuclear dioxin signaling. Nucleic Acids Res 2000;28(12):2286–2291.
178. Adachi J, *et al*. Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. J Biol Chem 2001;276(34):31475–31478.
179. Harper PA, *et al*. Detection and characterization of the Ah receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin in the human colon adenocarcinoma cell line LS180. Arch Biochem Biophys 1991;290(1):27–36.
180. Gonzalez FJ, Shah YM. PPARa: Mechanism of species differences and hepatocarcinogenesis of peroxisome proliferators. Toxicology 2008;246(1):2–8.
181. Katoh M, *et al*. Chimeric mice with humanized liver. Toxicology 2008;246(1):9–17.
182. Xie W, *et al*. Humanized xenobiotic response in mice expressing nuclear receptor SXR. Nature 2000;406(6794):435–439.
183. Ross J, *et al*. Human constitutive androstane receptor (CAR) and pregnane X receptor (PXR) support the hypertrophic but not the hyperplastic response to the murine nongenotoxic hepatocarcinogens phenobarbital and chlordane *in vivo*. Toxicol Sci 2010;116(2):452–466.
184. Scheer N, *et al*. *in vivo* responses of the human and murine pregnane X receptor to dexamethasone in mice. Drug Metab Dispos 2010;38(7):1046–1053.
185. Wang ET, *et al*. Alternative isoform regulation in human tissue transcriptomes. Nature 2008;456(7221):470–476.
186. David CJ, Manley JL. The search for alternative splicing regulators: new approaches offer a path to a splicing code. Genes Dev 2008;22(3):279–285.
187. Sammeth M, Foissac S, Guigo R. A general definition and nomenclature for alternative splicing events. PLoS Comput Biol 2008;4(8):e1000147.
188. Miles JS, *et al*. A novel human cytochrome P450 gene (P450IIB): chromosomal localization and evidence for alternative splicing. Nucleic Acids Res 1988;16(13):5783–5795.
189. Bertilsson G, *et al*. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. Proc Natl Acad Sci U S A 1998;95(21):12208–12213.
190. Dotzlaw H, *et al*. The human orphan receptor PXR messenger RNA is expressed in both normal and neoplastic breast tissue. Clin Cancer Res 1999;5(8):2103–2107.

191. Fukuen S, *et al*. Identification of the novel splicing variants for the hPXR in human livers. Biochem Biophys Res Commun 2002;298(3):433–438.
192. Lin YS, *et al*. The major human pregnane X receptor (PXR) splice variant, PXR.2, exhibits significantly diminished ligand-activated transcriptional regulation. Drug Metab Dispos 2009;37(6):1295–1304.
193. Savkur RS, *et al*. Alternative splicing within the ligand binding domain of the human constitutive androstane receptor. Mol Genet Metab 2003;80(1–2):216–226.
194. Auerbach SS, *et al*. Alternatively spliced isoforms of the human constitutive androstane receptor. Nucleic Acids Res 2003;31(12):3194–3207.
195. Lamba JK, *et al*. Expression of constitutive androstane receptor splice variants in human tissues and their functional consequences. J Pharmacol Exp Ther 2004;311(2):811–821.
196. Auerbach SS, *et al*. Retinoid X receptor-alpha-dependent transactivation by a naturally occurring structural variant of human constitutive androstane receptor (NR1I3). Mol Pharmacol 2005;68(5):1239–1253.
197. Auerbach SS, *et al*. CAR2 displays unique ligand binding and RXRalpha heterodimerization characteristics. Drug Metab Dispos 2007;35(3):428–439.
198. DeKeyser JG, *et al*. Di(2-ethylhexyl) phthalate is a highly potent agonist for the human constitutive androstane receptor splice variant CAR2. Mol Pharmacol 2009;75(5): 1005–1013.
199. Eichelbaum M, Ingelman-Sundberg M, Evans WE. Pharmacogenomics and individualized drug therapy. Annu Rev Med 2006;57,: 119–137.
200. Ingelman-Sundberg M. Implications of polymorphic cytochrome P450-dependent drug metabolism for drug development. Drug Metab Dispos 2001;29(4 II):570–573.
201. Arinç E. The role of polymorphic cytochrome P450 enzymes in drug design, development and drug interactions with a special emphasis on phenotyping. J Mol Catal, B Enzym 2010;64(3–4):120–122.
202. Ingelman-Sundberg M, *et al*. Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoepigenetic and clinical aspects. Clin Pharmacol Ther 2007;116(3):496–526.
203. Ingelman-Sundberg M. Polymorphism of cytochrome P450 and xenobiotic toxicity. Toxicology 2002;181–182,: 447–452.
204. Johnson AD, Wang D, Sadee W. Polymorphisms affecting gene regulation and mRNA processing: broad implications for pharmacogenetics. Clin Pharmacol Ther 2005;106(1):19–38.
205. Wang XD, *et al*. Impact of the haplotypes of the human pregnane X receptor gene on the basal and St John's wort-induced activity of cytochrome P450 3A4 enzyme. Br J Clin Pharmacol 2009;67(2):255–261.
206. Zhang B, Xie W, Krasowski MD. PXR: a xenobiotic receptor of diverse function implicated in pharmacogenetics. Pharmacogenomics 2008;9(11):1695–1709.
207. Lamba V, *et al*. Genetic predictors of interindividual variability in hepatic CYP3A4 expression. J Pharmacol Exp Ther 2010;332(3):1088–1099.